INFLAMMATORY MECHANISMS IN FOCAL CEREBRAL ISCHAEMIA

Jennifer F. McCarter B.Sc. (Hons)

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ABSTRACT

Stroke is a complex pathophysiological process and the role of inflammation in its initiation and resolution has been much debated. Inflammation is now emerging as a contributor in the development of ischaemic brain damage. The use of anti-inflammatory strategies to reduce damage and improve functional outcome of stroke patients may be valuable in the treatment for a condition that currently has no effective treatment. The exact contribution of the inflammatory response and the involvement of the various components of the immune system are still under investigation.

In this thesis, focal cerebral ischaemia was induced in three animal models in an attempt to investigate the contribution of the inflammatory response. The rat monofilament model of middle cerebral artery (MCA) occlusion, considered by some to be a pro-inflammatory model, was set up for the first time in Edinburgh and validated as suitable model for further investigation. Permanent and transient models were established to allow the evaluation of possible reperfusion injury. Both monofilament models were compared with the Endothelin-1 model already established and routinely in use in the laboratory. Analysis of the volume of damage and distribution of the lesion revealed no differences between the three models. However, this observation did not in itself rule out the possibility of different pathophysiological mechanisms in the three models that ultimately resulted in apparently similar sized lesions.

FK506, a potent neuroprotectant widely used experimentally, exhibited different neuroprotective efficacies. In all models, FK506 significantly reduced the overall volume of both damage and oedema. The majority of the neuroprotection was observed in the cortex although striatal protection was seen in the transient rat monofilament model. The neuroprotection observed in the transient monofilament model was approximately twice that

seen in the permanent model and similar to that in the Endothelin-1 model suggesting distinct pathways that lead to cell death. Data for FK506 administration in the mouse monofilament model demonstrated neuroprotection for the first time in this species was included as an interesting comparison with the rat data.

In keeping with the investigation of inflammation in cerebral ischaemia, it was proposed that FK506 neuroprotection was in part mediated through modulation of the inflammatory response. The response of the microglia, the resident immune cells of the central nervous system was examined following FK506 administration. Although the drug appeared to have a noticeable effect the activation state of the microglia, the response was not consistent and difficult to quantify by histological methods. Microglial cultures were established to investigate the effect of FK506 in a less complex system. Ramified microglial cultures were established but the analysis of microglia *in vitro* by morphology also proved difficult and another method of assessing activation of the cells was pursued. The microglia are known to secrete noxious stimuli when activated amongst which are the pro-inflammatory cytokines. IL-1β, IL-6 and TNFα gene expression was investigated to assess microglial activation. Lipopolysaccharide treated animals and treated microglial preparations were used initially to refine the use of multiplex polymerase chain reaction (MPCR) analysis of gene products. This was extended to tissue from both monofilament models. IL-1β, IL-6 and TNFα were detected in the cortex and striatum when measured at 3 and 24 hrs post occlusion and showed an earlier cytokine response where reperfusion occurred. It is suggested that the early cytokine response is associated with the endogenous inflammatory cells. Western analysis experiments were performed to verify the presence of the corresponding cytokine proteins with little success. The recent availability of improved cytokine antibodies enabled the examination of cytokines (IL-1β and TNFα) in ischaemic by enzyme linked immunosorbant assay (ELISA). No TNFα response was detected despite the presence of mRNA. IL-1β was detected at 3 and 24 hrs post-insult with greater expression at 24 hrs. It

may be speculated that this increased expression at the later time is related to the peripheral inflammatory cell infiltration. There was no difference in expression levels between models and FK506 had no affect on the cytokine expression.

In summary, the re-introduction of blood to ischaemic tissue appears to alter the response of the individual cells although this does not change their ultimate fate. In instances where reperfusion is established, the tissue appears to be more amenable to neuroprotection by FK506. It is suggested that this is associated with the blockade of the endogenous inflammatory mechanisms that respond acutely to an ischaemic insult.

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TO MY MUM,

MY BROTHER IAN,

AND THE MEMORY OF MY DAD

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My greatest thanks however and all my love goes to my Mum – 'We did it, Ma!'

DECLARATION

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

Signed _____

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PUBLICATIONS ARISING FROM THESIS

PAPERS

McCarter, J.F., McGregor, A.L., Jones, P.A., Sharkey J. (2000) FK506 protects brain tissue in animal models of stroke. *Transplantation Proceedings.* Submitted.

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BOOK CHAPTERS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABC	avidin-biotin complex
ACA	anterior communicating artery
ara-c	cytosine β-D-arabino-furanoside
ATP	adenosine triphosphate
ANOVA	analysis of variation
AP-1	activating protein 1
Bax	Bcl-2 associated protein X
BBB	blood brain barrier
BCA	bicinchoninic acid
Bcl-2	B cell lymphoma-2
BME	Basal Medium Eagle
bp	base pairs
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
Ca^{2+}	calcium ion
cAMP	cyclic AMP
CaN	calcineurin
caspase	cysteine aspartase
CCA	common carotid artery
cDNA	copy DNA
CK	creatine kinase
CO_2	carbon dioxide
CsA	cyclosporin A
CSF	cerebrospinal fluid

CVA	cerebrovascular accident
DAB	diaminobenzidine
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease 1
dNTPs	deoxyribonucleoside triphosphates
DPX	distrene, plasticiser and xylene slide mounting medium
EB	extraction buffer
ECA	external carotid artery
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
EEA	excitatory amino acids
EGTA	ethylene glycol-bis (β-aminoethyl ether) *N,N,N,N* tetra-acetic acid
e/i/nNOS	endothelial / inducible / neuronal nitric oxide synthase
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Et-1	endothelin-1
EtBr	ethidium bromide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FKBP/s	FK506 binding protein/s
g	gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary associated protein
GM-CSF	granulocyte macrophage colony stimulating factor

H^+	hydrogen ion
H_2O_2	hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
hr/s	hour/hours
i.p.	intraperitoneal
i.v.	intravenous
ICA	internal carotid artery
ICAM	intracellular adhesion molecule-1
ICE	interleukin converting enzyme
IEG	immediate early gene
IgG	immunoglobulin G
Iκ-B	inhibitor of kappa light chain gene enhancer in B cells
IL-1β	interleukin-1β
IL-1ra	interleukin-1 receptor antagonist
IL-2	interleukin-2
IL-6	interleukin-6
IP_3	inositol 1,4,4, trisphosphate
IP_3R	inositol 1,4,4, trisphosphate receptor
K^+	potassium ion
kDa	kilodalton
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MABP	mean arterial blood pressure
MCA	middle cerebral artery
MCID	microcomputer imaging device
mg	milligram

MHC	major histocompatibilty complex
min	minute
ml	millilitre
µl	microlitre
mm	millimetre
µM	micromolar
M-CSF	macrophage colony stimulating factor
MPCR	Multiplex polymerase chain reaction
MTP	mitochondrial permeability transition pore
MQ	MilliQ water
mRNA	messenger RNA
Na^+	sodium ions
NF-AT	nuclear factor of activated T cells
NFG	nerve growth factor
NFκ-B	nuclear factor of kappa light chain gene enhancer in B cells
ng	nanogram
NGF	nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
O_2	oxygen
OA	occipital artery
OGD	oxygen-glucose deprivation
PBS	phosphate buffered saline
PCr	phosphocreatine
PCR	polymerase chain reaction
PDVF	polyvinylidene fluoride

PET	positron emission tomography
PFA	paraformaldehyde
PLP	periodate-lysine-paraformaldehyde
PMF	permanent monofilament
PMNLs	polymorphonuclear leukocytes
PMSF	phenyl-methyl-sulfonyl-fluoride
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
RyR	ryanodine receptor
SD	Sprague Dawley
SDS	sodium dodecyl sulphate
sem	standard error of the mean
STA	superior thyroid artery
TAE	Tris acetate EDTA buffer
TBST	Tris Buffered Saline-Tween 20 (0.5 %) buffer
TGF-β	transforming growth factor-β
Tm	melting temperature
TMF	transient monofilament
TNFα	tumour necrosis factor α
tPA	tissue plasminogen activator
Tris	Tris-[hydroxymethyl]-aminomethane
TTC	2,3,5-triphenyltetrazolium chloride
TUNEL	terminal deoxynucleotidyl transferase nick end labelling
Tween 20	polyoxyethylene-sorbitan monolaurate
Zn^{2+}	zinc

'The words are the words of a master, but we are not forced to swear by them. Instead we are to be borne wherever experiment drives us.'

Horace

CHAPTER 1

General Introduction

INCIDENCE & EPIDEMIOLOGY OF STROKE

Stroke is defined by the World Health Organisation as "rapidly developing signs of focal (or global) disturbance of cerebral function lasting longer than 24 hours with no apparent non-vascular cause" (Thorvaldsen *et al.*, 1995). Thus defined, it is the most common disabling and lethal neurological disease of adult life (Dyken *et al.*, 1984) and is still the third leading cause of death among adults after cancer and myocardial infarction (Gorelick, 1995; Office for National Statistics Mid-year estimates 1994). Worldwide there are approximately 4.6 million deaths attributed to stroke each year (Bonita & Beaglehole, 1995). The most comprehensive data on stroke comes from the United States (US) where the incidence is approximately 250-400 in 100 000 (750 000 per year) with a mortality rate of 30 %. Recent figures from the American Heart Association show that someone suffers a stroke every 53 seconds with a stroke related death occurring every 3.3 minutes (min) (American Heart Association, 2000). Over 4 million survivors live with debilitating consequences of the disease. By the year 2050 it is expected that there will be over a million strokes per year in the US (Barone & Feuerstein, 1999; Dirnagl *et al.*, 1999).

In comparison, epidemiological studies in the United Kingdom (UK) indicate that there are approximately 60 000 deaths from stroke each year with 100 000 people experiencing a first time stroke, 10 000 of these are under retirement age (Office for National Statistics Mortality Statistics, 1997). In England and Wales, as with the rest of the world, stroke is the single

largest cause of severe disability with over 300 000 people being affected at any one time (Office for National Statistics Mid-year Estimates 1994; Prevelance from Geddes, 1996). Stroke patients occupy around 20 % of all acute hospital beds and 25 % of long-term beds (Wade, 1994), costing the National Health Service an estimated £2.3 billion that is forecast to rise around 30 % by the year 2023 (Department of Health, Burdens of Disease 1996). In England, 1 in every 500 deaths is stroke related and these deaths account for 8 % of all deaths in men and 13 % of deaths in women (Health Survey for England 1995). In Scotland, the statistics from the Clinical Outcome Indicators Report 1999 for the period 1995-1998 show that 29 439 patients presenting with a stroke were admitted and 22 328 patients (76 %) survived for 30 days following emergency admission (Scottish Executive Health Department Clinical Outcomes Indicators Report 1999).

The term stroke, or cerebral ischaemia, is generally used to describe the cessation of oxygen and glucose (substrates) supplied to a brain region as a consequence of transient or permanent interruption of blood supply causing neuronal cell death (Edvinsson *et al.*, 1993). The concept of ischaemia is not particularly useful as the condition is better described by the term oligaemia, which by definition implies an organ or some portion receives a reduced blood supply and therefore insufficient oxygen and suitable substrates (Graham, 1985). Cerebral ischaemia is commonly divided into two major categories. The first and most common, accounting for 80 % of cerebrovascular accidents (CVA) is termed ischaemic stroke (Ringelstein & Nabavi, 2000). This classification can be further sub-divided into global ischaemia, seen in conditions such as cardiac arrest where there is cessation of blood flow from the heart and focal ischaemia, which is observed following a localised disruption of the blood supply to the brain via the occlusion of a branch or stem of an intracranial vessel (Ter Horst & Korf, 1997). The occlusion of a cerebral vessel that results in the disruption of the blood supply can be caused either by a thrombosis (clot formation within the vessel), embolism (movement of a clot from elsewhere in the body) or stenosis (a severe narrowing

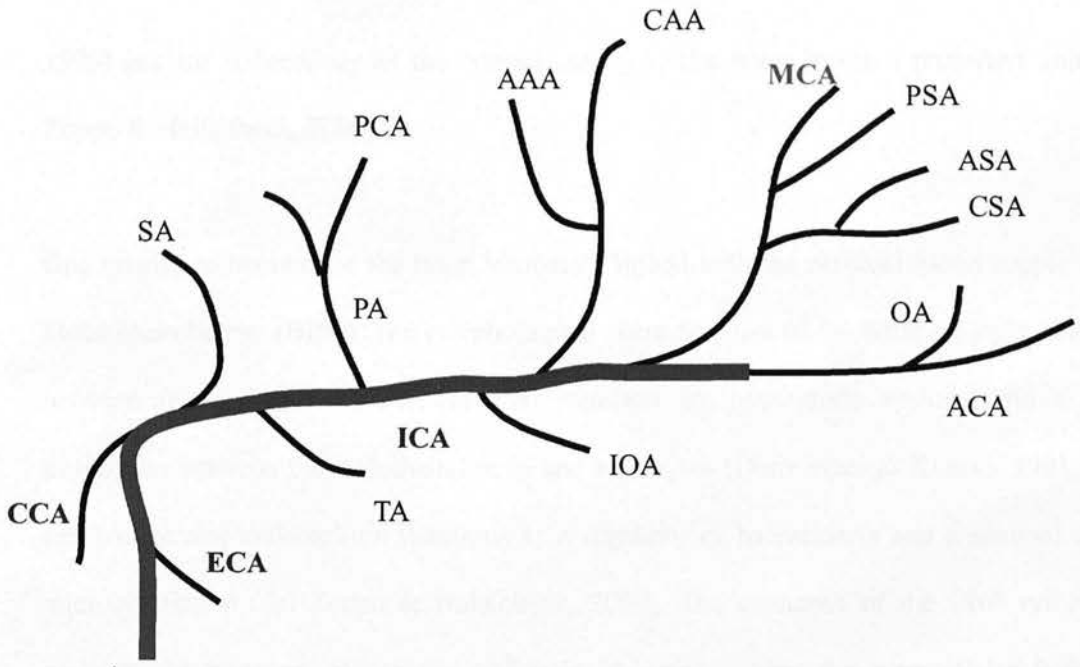
of an artery in or leading to the brain (National Institute of Neurological Disorders and Stroke 2000). In humans, occlusion of the middle cerebral artery (MCA) is the most common underlying cause of ischaemic stroke (Nedergaard, 1988). Ischaemic incidents that are resolved within 24 hours are termed transient ischaemic attacks and may be indicative of a major impending (Moncayo *et al.*, 2000; Ter Horst & Korf, 1997). Haemorrhagic stroke is the second classification of ischaemic stroke and accounts for approximately 20 % of CVAs and is the consequence of a ruptured intracranial vessel (Ringelstein & Nabavi, 2000). Bleeding from a ruptured vessel can occur intracerebrally into the brain parenchyma or into the subdural or subarachnoid spaces surrounding the brain. In the UK, 80 % of initial strokes suffered are ischaemic in nature, 10 % are due to primary intracerebral haemorrhage and 5 % are caused by subarachnoid haemorrhage. In 5 % of stroke cases, the type of stroke is not clear. Among patients who suffer an ischaemic stroke, 3-4 % experience another ischaemic episode shortly after the initial insult and 10 % of these patients die within the first 2-4 weeks. Approximately 10 % have a recurrence of stroke within a year. Among patients who have an intracerebral haemorrhage, 50 % die within 30 days and 60 % within one year (The Stroke Association 2000).

BRAIN STRUCTURE & CEREBRAL CIRCULATION

Brain parenchyma consists of two types of cells, neurones and neuroglia (glia). In the human brain, there are approximately 10^{10} neurones and 10^{11} supporting glial cells that are supplied by a cerebrovascular network consisting of endothelial cells and smooth muscle. Both morphology (e.g. pyramidal neurones) and neurochemistry (e.g. serotonergic neurones) are used to classify neurones whereas the glia are divided into three classes. Oligodendrocytes are responsible for formation and maintenance of myelin, the astrocytes provide metabolic support for the neurones and modulate the extracellular environment and the microglia are

considered to be the resident inflammatory cells of the central nervous system (CNS) (Moller *et al.*, 2000; Perry *et al.*, 1993; Raivich *et al.*, 1999; Streit, 1993). One unique aspect of the cerebral circulation is the absence of a lymphatic system that is found in most other organs. Substances of brain origin that are deposited in the lymph nodes are believed to have been transferred from the cerebrospinal fluid to the extracranial fluid through the cribiform plate and the spaces around the trunks of emerging cranial and spinal nerves (Scremin, 1995).

The anatomy of the arterial supply of the cerebral hemispheres in the rat is similar to that in humans (Yamori *et al.*, 1976). Each species has an anterior, middle and posterior cerebral artery supplying each hemisphere. These vessels are all derived mainly from the internal carotid arteries (ICA) but connected via an azygous anterior cerebral and posterior communicating artery to form a modified Circle of Willis (***Figure 1***). The vertebral arteries fuse to form the basilar artery that supplies posterior structures of the brain via the posterior cerebral artery. The middle cerebral artery of the rat runs laterally over the surface of the olfactory tract before branching to supply the cerebral cortex where distally the branching is more variable. The medial lenticulostriate arteries branch from the MCA and supply the posterior caudate putamen (striatum) and olfactory tract (Yamori *et al.*, 1976). The baseline blood flow through the striatum is substantially lower than that the cortical flow and consequently flow disturbances are more likely to produce injury (del Zoppo & Hallenbeck, 2000). In the rat however, the local cerebral blood flow of the striatum is similar to the overlying cortex (Sakurada *et al.*, 1978). The anterior striatum is supplied by the lateral striate branches and receives a medial supply from Hubner's arteries from the anterior cerebral (Tamura *et al.*, 1981a).



CEREBRAL ARTERIES

AAA	anterior amygdaloid	IOA	internal ophthalmic
ACA	anterior cerebral	**MCA**	**middle cerebral artery**
ASA	anterior striates	OA	olfactory
CAA	cortico-amygdaloid	PA	posterior communicating
CCA	**common carotid**	PCA	posterior cerebral
CSA	cortico-striates	PSA	posterior striates (lenticulate)
ECA	**external carotid**	SA	stapedial (pteriopalatine)
ICA	**internal carotid**	TA	trigeminal

Figure 1. Simplified diagram showing the cerebral circulation of the rat. Diagram additionally shows the placement of a nylon monofilament to occlude the middle cerebral artery.

The anterior choroidal arteries supply the choroid plexus and the hippocampus and the posterior choroidal arteries supply caudal structures such as the cerebellum. In the rat brain, as in many other species, there are numerous arterial/venous anastomoses that make it almost impossible to produce complete localised ischaemia by occlusion of a blood vessel (Scremin,

1995) and the redundancy of the arterial supply to the brain serves a protective role (del Zoppo & Hallenbeck, 2000).

One crucial component of the brain intimately linked with the cerebral blood supply is the blood brain barrier (BBB). The morphological characteristics of the BBB are tight junctions between the endothelial cells, minimal transport by pinocytotic vesicles and a close interaction between the endothelial cells and astrocytes (Dermietzel & Krause, 1991). The cerebrovascular endothelium functions as a regulator of haemostasis and a sentinel of the microcirculation (del Zoppo & Hallenbeck, 2000). The existence of the BBB effectively prevents the transport of proteins and polar substances from the extracellular fluid and protects the brain for alterations in systemic fluctuations of hormones (Dalton Dietrich, 1999). There are however several areas of the brain such as the area postrema and some parts of the hypothalamus where the BBB is incomplete. These areas are associated with the functioning and feedback mechanisms of the neuroendocrine system. In these areas, the capillaries have fenestrations and the endothelial cells contain a large number of pinocytotic vesicles (Heistad & Kontas, 1983). In addition to the physical barrier that exists, there is also a chemical or enzymatic barrier (e.g. monoamine oxidase in the endothelial cells) that contributes to the maintenance of brain homeostasis. Small hydrophobic molecules are able to cross the BBB because of the lipophilic nature of the membranes. Hydrophilic substances such as proteins and saccharides cannot readily diffuse through the membrane and therefore require specific transport mechanisms (Dermietzel & Krause, 1991) such as the multi-drug resistance receptor 1, which is the transporter for drugs such as FK506 (Yokogawa *et al.*, 1999). Following an ischaemic insult the integrity of the BBB may become disrupted allowing exposure of brain tissue to unregulated levels of blood borne substances. Additionally, BBB disruption contributes to damage and the formation of oedema within the ischaemic tissue (Edvinsson *et al.*, 1993; Heistad & Kontas, 1983).

CONTROL OF THE CEREBRAL CIRCULATION

Two notable characteristics of the cerebral circulation are the ability of the brain to maintain relatively constant cerebral blood flow (CBF) over a wide range of perfusion pressures, termed autoregulation (Harper, 1966) and the ability to dynamically alter local blood flow to accommadate for dynamic changes in metabolic activity of the brain, termed flow metabolism coupling.

Autoregulation, first described in the brain by Fog in the 1930s, protects the brain against changes in systemic blood pressure (Fog, 1939). In man, cerebral blood pressure remains constant within the mean arterial blood pressure (MABP) range of 65-140 mm Hg (Strandgaard & Paulson, 1984). Beyond these limits, autoregulatory mechanisms of the brain fail. At MABP above 140 mm Hg there is a forced dilation of the cerebral blood vessels, cerebral blood flow (CBF) rises and oedema eventually results (hypertensive encephalopathy). Beyond the lower limit, CBF falls and signs on hypoxia becomes evident (Harper, 1990; Heistad & Kontas, 1983) (***Figure 2***). It is important to note that in ischaemic tissue the autoregulatory response of the brain is lost.

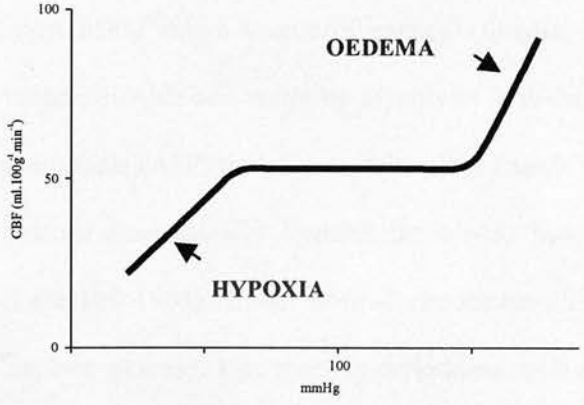


Figure 2. Autoregulation curve (Harper, 1990).

The concept of flow metabolism coupling predates autoregulation by some 40 years. Roy & Sherrington, as far back as 1890, suggested a link between vascular and functional activity in the brain and related it to the chemical products of cerebral metabolism (Roy & Sherrington, 1890). Although the precise mechanisms that drive flow metabolism coupling have not been definitively established and number of potential molecules have been proposed including O_2, H^+, NO and adenosine (Harper, 1990; Matsuyama, 1997; Sokoloff & Kety, 1960). The functional activity of any brain region is intrinsically linked with energy consumption in that region imposed by normal physiological activity. In man, the normal flow rate is approximately 1.5 ml.μmol glucose consumption^{-1}.min^{-1}. Under ischaemic conditions, as with the autoregulatory response of the brain, flow metabolism coupling is compromised.

PATHOPHYSIOLOGY OF CEREBRAL ISCHAEMIA

The brain, although it only comprises 2-3 % of total body weight, receives 15 % of total cardiac output and consumes 20 % of oxygen and 25 % of glucose circulating in the blood (Graham, 1985). In humans, it accounts for 25 % of basal metabolism with a metabolic rate 3.5 times higher than that of other primate species (Lee *et al.*, 2000). Calculation of the respiratory quotient of the brain gives a value of one implying that oxidative catabolism of glucose is the most likely single source of energy. Glucose extracted from the blood is metabolised to carbon dioxide and water by glycolysis with one glucose molecule yielding 42 adenosine triphosphate (ATP) molecules. Like other tissues of the body, the brain is able to metabolise glucose anaerobically forming lactic acid but the energy yield from this process is small (Siesjo, 1984). Under normal circumstances, no substance found in the circulation can replace glucose. However, in conditions such as starvation, the brain may oxidise ketone bodies when blood concentrations of these molecules is increased above normal (Siesjo, 1978).

One third of the energy generated by oxidative metabolism of glucose is used for the maintenance of synaptic transmission, one third for cell homeostasis and the remaining third for the maintenance of structural integrity (Back, 1998). Due to the large metabolic requirement of the brain (60 W) and its very limited capacity for substrate storage, the survival of cells in the organ is dependent on the continuous and adequate supply of oxygen and nutrients. The interruption of the blood supply that occurs in conditions such as cerebral ischaemia which causes an energy crisis within the tissue can lead to neuronal cell death within minutes (Astrup *et al.*, 1981).

VIABILITY THRESHOLDS

CBF in man has been calculated to be 50 ml.100 g^{-1}.min^{-1} which remains constant although there are continuous alterations in local blood flow that adjust in response to the metabolic needs of different brain regions (Harper, 1990). In the rat, CBF is approximately 150 ml.100 g^{-1}.min^{-1} (McCulloch *et al.*, 1982) where the higher CBF value in the rat compared with the human value reflects the difference in neuronal density between the species (Hossmann, 1994).

Astrup *et al.* (1977) recognised the concept of viability thresholds in cerebral ischaemia and differentiation in critical blood flow rates required for electrical failure versus membrane failure (Astrup *et al.*, 1977). Although the determination of thresholds has been conducted in a number of different species making it difficult to directly compare absolute values, there is a distinct hierarchy of susceptibility of energy dependent cellular functions (Hossmann, 1994) (***Figure 3***). The threshold for depression of protein synthesis, oedema formation and acidosis occurs before the threshold of electrical failure. Protein synthesis is most sensitive to a decline in CBF. Glucose utilisation transiently increases as flow rates decrease and then

decline sharply corresponding to lactate accumulation and acidosis. Below this level, ATP and phosphocreatine (PCr) levels begin to decline (Hossmann, 1994). When CBF rates drop to one third of normal levels, there is loss of electrical excitability. Membrane failure and ion homeostasis is lost when CBF falls below one quarter of normal values (Siesjo, 1992a). At this level of CBF, ion fluxes result in the death of neurones in minutes (Sweeney *et al.*, 1995). In the rat, CBF rates below 25 ml.100 $mg^{-1}.min^{-1}$ produces well-demarcated tissue damage. This threshold value is higher than that of larger species that is again most likely explained by the higher neuronal density in the rat brain (Back, 1998).

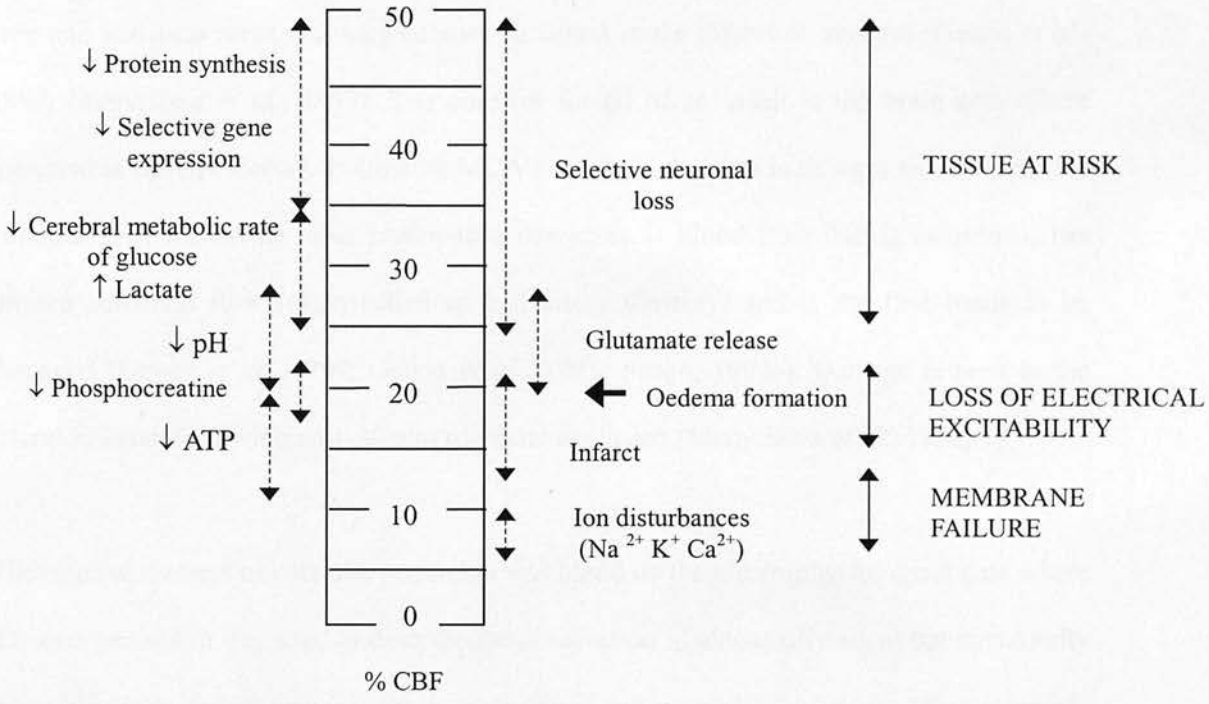


Figure 3. Viability thresholds of cerebral ischaemia (Hossmann, 1994; Siesjo, 1984; Siesjo 1992a).

CORE, PENUMBRA & REPERFUSION INJURY

In discussions regarding focal cerebral ischaemia there are a number of terms that are used to describe the damage that follows the occlusion of a blood vessel and are best clarified. The term lesion is defined in the Oxford English Dictionary as damage, injury or morbid change in the functioning or texture of an organ. Infarct is defined as a small area of dead tissue caused by an inadequate blood supply and thus the process of infarction is the events that lead to the dead tissue or area of coagulation necrosis that develops (Garcia & Liu, 1996).

It has long been widely believed that an ischaemic lesion consists of a densely ischaemic core and perifocal areas that may become recruited in the infarction process (Garcia *et al.*, 1995; Memezawa *et al.*, 1992). The core (or focus) of an insult is the brain area where irreversible damage occurs. Following MCA occlusion, the core is thought to lie within the striatum as it shows the most pronounced decreases in blood flow during ischaemia, has limited collateral flow (often called an end artery territory) and is the first tissue to be damaged (Dewar *et al.*, 1999; Garcia *et al.*, 1995; Siesjo, 1992a). Damage is seen in the lateral striatum following only 30 min of vessel occlusion (Memezawa *et al.*, 1992).

The original concept of core and penumbra was based on the electrophysiological data where the term penumbra was used to describe the observation of electrically silent but structurally intact neurones found between the necrotic core and normal brain tissue (Symon *et al.*, 1977). The area of compromised tissue resembled the partly illuminated area around the complete shadow of the moon in full eclipse, hence the name penumbra (Hossmann, 1994). The peri-infarct zone contains cells that are at risk and survival is dependent on severity and duration of vessel occlusion (Hallenbeck & Dutka, 1990). Astrup *et al.* (1981) defined the penumbra as brain tissue perfused at a level between thresholds of functional impairment and

loss of morphological integrity that would have the capacity to recover if perfusion was re-established (Astrup *et al.*, 1981). Although the majority of the tissue damage occurs within the first few hours following cerebral ischaemia, it is well established that penumbral tissue may continue to deteriorate at a later time (Garcia & Kamijyo, 1974; Heiss *et al.*, 1999). The concept of core and penumbral tissue has led to the view that the tissue of the penumbra can be salvaged by administration of pharmacological agents or by relatively prompt reperfusion (***Figure 4***) (Memezawa *et al.*, 1992). This concept, in principle provides a so-called 'therapeutic window of opportunity' for intervention but does require knowledge of the temporal development of the lesion and suitable target identification (Bartus *et al.*, 1995). There are physiological (blood flow) and biochemical differences between core and penumbra tissues and also different responses to neuroprotectant compounds (Ginsberg *et al.*, 1996; Zhang *et al.*, 1995). The ischaemic penumbra is a dynamic tissue structure (Back, 1998) which Ginsberg & Pulsinelli (1994) called 'an evolving zone of bioenergetic upheaval' (Ginsberg & Pulsinelli, 1994). Siesjö (1992) suggested that recruitment of tissue in the penumbra may occur as cell death continues until well-perfused tissue is encountered or alternatively small islands of necrotic cells develop affecting neighbouring cells to ultimately coalesce and produce a large infarct (Siesjo, 1992a). Data from both cats and monkeys suggest that there is a gradual development of the lesion during the first 4-8 hours (hrs) although there is a large amount of variability. This variability in experimental animals may reflect differences in collateral blood supply of individual animals and differences in species. Slow evolution of the penumbra in the cortex may be a consequence of collateral blood flow (Memezawa *et al.*, 1992). It should be noted that the anatomical and temporal extent of ischaemic penumbra is not as consistent in man as the penumbra in animals, even when using the same technology (Akins *et al.*, 1996; Baird & Warach, 1998; Heiss & Graf, 1994). The peri-infarct region of the lesion in rats is larger than that seen in human and sub-human primates and may explain the moderate results obtained with potential therapies when administered in the clinical setting (Siesjo, 1992a). In addition, human strokes tend to

be more variable, both in infarct size and neurological deficit (Lutsep & Clark, 1999). A further point to consider when discussing the development of ischaemic damage is the effect of physiological variables such as CO_2 levels, blood pressure and temperature on outcome following an insult. These variables can only be controlled under experimental conditions.

An ideology that is closely allied to the concept of the core and penumbra of an ischaemic lesion is concept of reperfusion injury. Reperfusion injury is well recognised in the heart and in other organs that are susceptible to ischaemic damage (Grech *et al.*, 1995; Werns & Lucchesi, 1990; Zwacka *et al.*, 1998) and thus potentially it may occur in the ischaemic brain. Del Zoppo *et al.* (2000) recently recognised that reperfusion injury in the brain is a widely held concept but commented that there is little data to support this claim. They did however acknowledge that under some circumstances an increase in ischaemic damage may seen (del Zoppo *et al.*, 2000). Experimental data, clinical observations and data available from other organs suggest that reperfusion results in a smaller infarct volume (del Zoppo *et al.*, 2000). The restoration of the blood to the ischaemic tissue, on one hand, halts the processes leading to cell death but may alternatively lead to further damage in compromised tissue, depending on both the duration of ischaemia and efficiency of reperfusion (Marchal *et al.*, 1999; Park & Lucchesi, 1999). The return of blood to ischaemic tissue signals both the return of substrates vital for cellular metabolism and the removal of accumulated metabolic waste that is conducive to the restoration of normal metabolism. However, following transient occlusion of the MCA, the initial period of ischaemia leads to slowly maturing cell death despite the resumption of basic biochemical functions within the cell due to the return of blood to the ischaemic zone (***Figure 4***) (Kuroda & Siesjo, 1997). It is plausible that the development of lesion occurs because of depression of protein synthesis resulting from deranged cellular functioning, gradual accumulation of calcium (Ca^{2+}) intracellularly that contributes to mitochondrial dysfunction, generation of damaging free radicals and altered signalling within the cell. In addition to intracellular events, the return of blood to ischaemic

tissue may initiate an inflammatory response that may in itself cause the release of damaging molecules such as free radicals and pro-inflammatory cytokines and the infiltration of peripheral cells such as leukocytes and monocytes (Kuroda & Siesjo, 1997).

Recanalisation is a complex issue and does not necessarily equate with adequate tissue perfusion despite the return of blood to the area. Intricate phenomena (haemorrhage, oedema, vascular plugging) may lead to a 'no-reflow' situation (Marchal *et al.*, 1999). On the other hand, reperfusion may occur as a result of perfusion pressure though collateral vessels. The concept of no-reflow following an ischaemic insult is a debated topic with some authors questioning it occurrence (Kuroda & Siesjo, 1997; Li *et al.*, 1998). Positron emission tomography (PET) studies in humans suggest that early reperfusion is not detrimental and contradicts the experimental concept of reperfusion injury and supports the use of early thrombolysis (Marchal *et al.*, 1999). Impairment of microvascular reperfusion could occur due to narrowing of the blood vessels as a result of perivascular swelling and oedema causing raised intracranial pressure, a change in blood dynamics caused by fluid shifts in the damaged tissue or capillary plugging by cells such as leukocytes (Gartshore *et al.*, 1997; Hallenbeck & Dutka, 1990). Reperfusion may also affect the function of the arteries whose role is critical to maintaining cerebrovascular resistance. Cipolla *et al.* (1997) showed abnormal structure and function of the MCA with diminished myogenic reactivity and tone associated with longer reperfusion (Cipolla *et al.*, 1997).

In rats, Kaplan *et al.* (1991) reported that the maximal reperfusion window was 3 hrs following 30 or 60 min occlusion (Kaplan *et al.*, 1991). Memezawa *et al.* (1992) showed that 30 min occlusion caused infarction in the lateral striatum although some experimental animals did have cortical damage. A 60 min MCA occlusion produced extensive damage with both striatal damage and extensive cortical damage that was however smaller than a 120 or 180 min occlusion. They showed that reperfusion following occlusion was beneficial if

instituted between 90 and 120 post-occlusion but after 120 min, return of blood flow failed to salvage tissue. For optimal tissue salvage reperfusion needed to be established within 60 min of occlusion. Additional observations from this study proved tissue damage following 1 hour (hr) occlusion in a rat monofilament model was smaller that 24 hrs permanent occlusion, again supporting the notion that reperfusion following ischaemia is beneficial (Memezawa *et al.*, 1992). Data from studies in this laboratory using the Endothelin-1 (Et-1) model (considered to be a permanent occlusion) and the Endothelin-3 (Et-3) model (developed as a model of MCA occlusion with controlled reperfusion) showed no significant difference in the volume of damage between the two models implying no beneficial effect of reperfusion (Henshall, 1997). A study using PET to evaluate reperfusion injury in monkeys showed significantly greater damage following transient occlusion of the MCA compared with a permanent occlusion model with the increase damage observed in the cortex (Takamatsu *et al.*, 2000). In a series of experiments, Du *et al.* (1996) demonstrated that a mild focal insult (30 min) caused infarction after 3 days and led to a lesion that was indistinguishable from that induced by a severe (90 min) occlusion after 14 days. The authors suggested that the delayed development of the lesion was due to apoptotic cell death in the peri-infarct zone of the lesion (Du *et al.*, 1996). However, in a study that repeated these experiments using the Endothelin models, no difference in the volume of ischaemic damage was seen following a 30 min occlusion when assessed at 3 or 14 days suggesting apoptosis is unlikely to contribute to the lesion volume (Henshall, 1997).

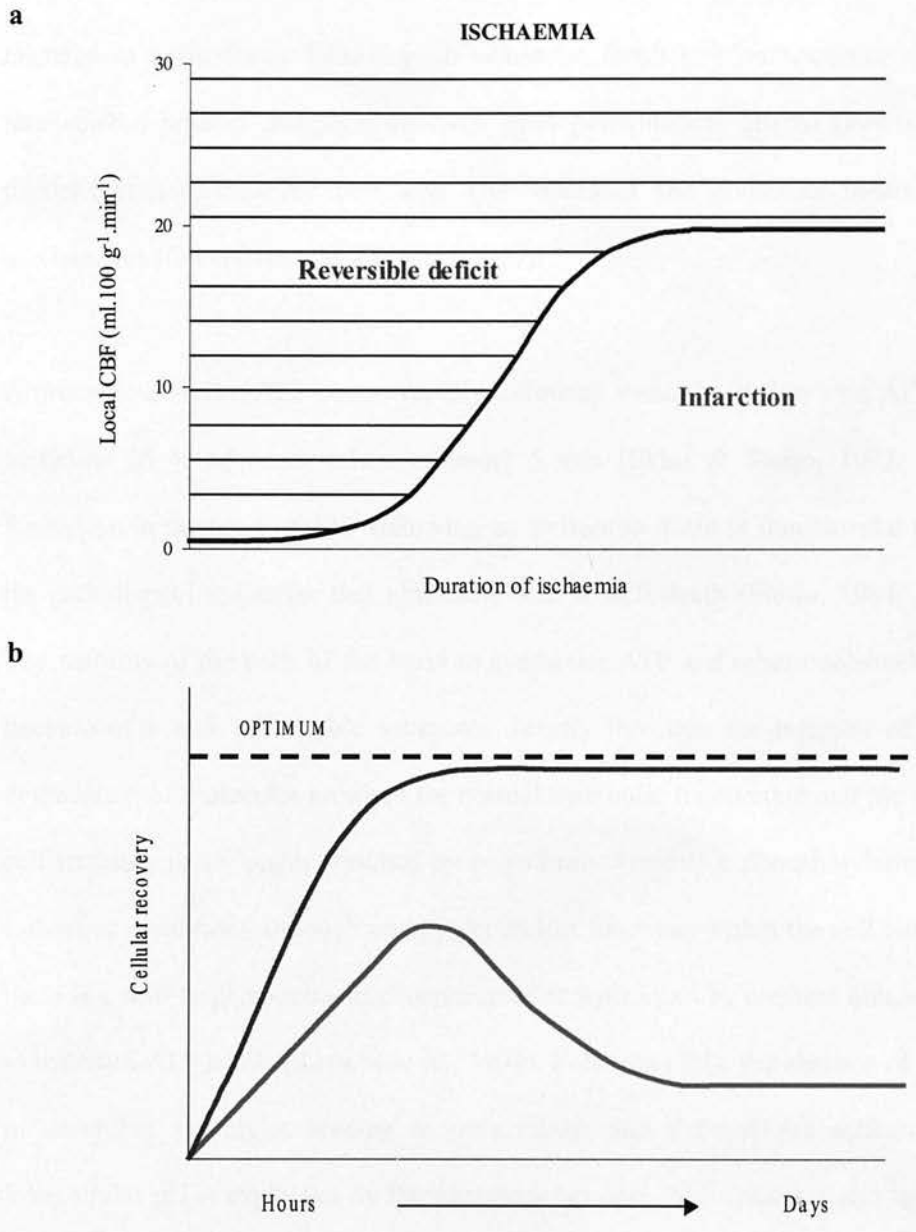


Figure 4. Levels of residual blood flow and duration of ischaemia (a) showing that lower levels of blood flow are tolerated for shorter than higher residual flow. The green curve (b) shows theoretically optimal reperfusion. The red curve denotes reperfusion injury (Halestrap *et al.*, 2000).

BIOCHEMICAL SEQUELAE OF ISCHAEMIA

Damage to brain tissue following an ischaemic insult is a consequence of a number of inter-related process including acidosis, lipid peroxidation, altered protein synthesis and phosphorylation, impaired perfusion, Ca^{2+} overload and glutamate mediated excitotoxic mechanisms (Obrenovitch & Richards, 1995).

A precipitous fall of ATP occurs rapidly following vessel occlusion with ATP levels falling to below 25 % of basal values in under 5 min (Eklof & Siesjo, 1972; Lipton, 1999). Reduction in the level of ATP following an ischaemic insult is fundamental to the initiation the pathological sequealae that ultimately lead to cell death (Siesjo, 1984; Siesjo, 1992a). The inability of the cells of the brain to synthesise ATP and other nucleoside triphosphates because of a lack of suitable substrates directly threatens the integrity of the cells. The degradation of molecules required for normal metabolic functioning and the maintenance of cell structure is no longer matched by resynthesis. Oxidative phosphorylation ceases under ischaemic conditions although energy dependent functions within the cell continue. Initially there is a shift to glycogen and phosphocreatine hydrolysis by creatine kinase (CK) in order to maintain ATP levels (Katsura *et al.*, 1994). Following this, the absence of glucose results in anaerobic glycolysis leading to intracellular and extracellular acidosis. The fall of intracellular pH is explained by the mismatch between ATP synthesis and hydrolysis as the metabolism changes. The drop in extracellular pH is a consequence of the generation of hydrogen (H^+) ions and their extrusion from the cells by membrane transporters (Martin *et al.*, 1994). The further reduction in ATP levels causes the failure of ion pumps that maintain membrane potential, disruption of homeostasis and leads to loss of function (Lipton, 1999).

Although discussed in sections in this thesis for clarity, the biochemical sequealae are a series of inter-related events that occur following the reduction of cerebral blood flow. The events in the cascade are complex and it is sometimes difficult to view the events individually and at all times one must be aware of the implications that a pathological change in one cellular function potentially has deleterious effects on a number of other functions. An overall schematic representation of the ischaemic cascade (***Figure 5***) gives a simplified flow diagram of the events following an ischaemic insult.

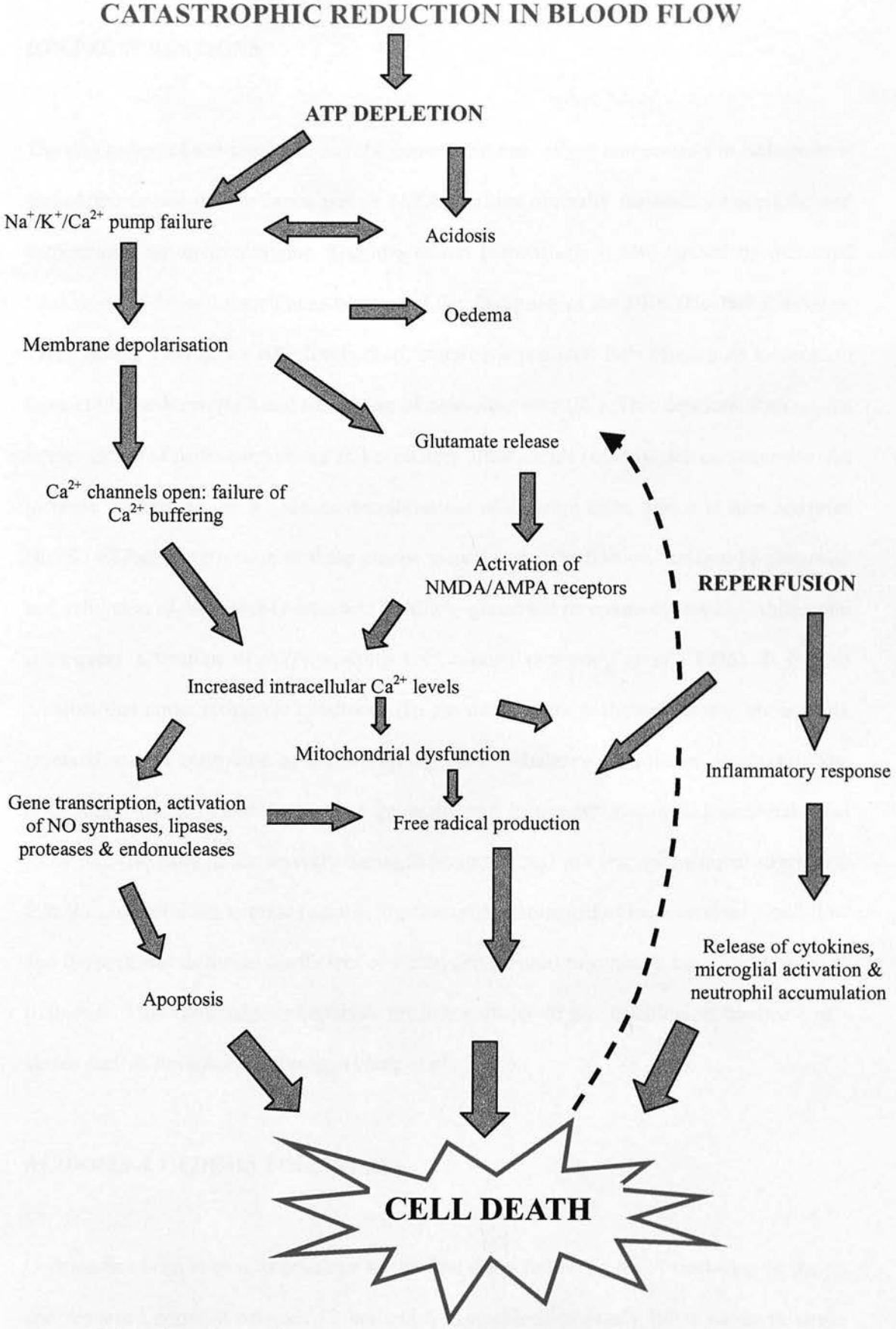


Figure 5. The ischaemic cascade.

ION PERTURBATIONS

The dissipation of ion gradients and the consequent loss of ion homeostasis in ischaemia is caused the failure of membrane pumps (ATPases) that normally maintain intracellular and extracellular ion concentrations. The loss of ion homeostasis is also caused by increased 'leakiness' of the cell membranes because of the disruption of the BBB (Heistad & Kontas, 1983; Siesjo, 1992a). As ATP levels drop, membrane potential falls causing an increase in intracellular sodium (Na^+) and the release of potassium ions (K^+). This depolarisation results in the release of neurotransmitters and excitatory amino acids (EEAs) such as glutamate. An increase in extracellular K^+ causes depolarisation of adjacent cells, which in turn activates Na^+/K^+ ATPases. Activation of these pumps increases ATP utilisation. Release of glutamate and activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors causes Ca^{2+} influx and subsequent activation of ATP-requiring Ca^{2+} pumps (Sweeney *et al.*, 1995). It is also possible that under ischaemic conditions the ion transporters in the membranes are actually reversed and so contribute to the worsening ionic imbalances (Phillis *et al.*, 2000). The observation that Na^+ ions accumulate gradually and incessantly during experimental focal ischaemia (but only in the severely damaged brain regions) has lead to the novel suggestion that Na^+ concentration in these regions, together with assessment of local cerebral blood flow and the apparent diffusion coefficient of water, can be used to estimate the time of onset of ischaemia. This knowledge is important regarding choice of pharmacological treatment of a stroke such as thrombolytic therapy (Wang *et al.*, 2000).

ACIDOSIS & OEDEMA FORMATION

Oedema has been shown to develope in the first 6 hrs following MCA occlusion in the rat and remained constant between 12 hrs and 3 days (Menzies *et al.*, 1993) whilst in stroke patients maximum oedema is seen at 72 hrs (Rosenberg, 1999). Intracellular pH begins to

fall by 0.5-1 units shortly after insult. This can be explained by the mismatch of ATP metabolism as the cells switch from aerobic to anaerobic metabolism, as the cells are unable to continue oxidative phosphorylation. The fall in extracellular pH is caused by the extrusion of H^+ ions and lactate (Martin *et al.*, 1994). The accumulation of lactate within the cells also contributes to the lowered pH intracellularly (Plum, 1983; Siesjo, 1992b; Rehncrona *et al.*, 1981). Energy failure and acidotic activation of transporters in the membranes causing an accumulation of Na^+ and Cl^- ions in the cell and with them osmotically obligated water. Tissue swelling can cause massive secondary damage by compression of the contralateral brain and other remote regions. This is one of the primary causes of mortality in both experimental models and human stroke sufferers (Witte *et al.*, 2000).

Betz *et al.* (1994) demonstrated that Na^+ ions were principally involved in oedema formation in the first 3-6 hrs following MCA occlusion (Betz *et al.*, 1994). The cells attempt to rectify the intracellular pH at the expense of their own volume regulation (Siesjo, 1992b). The increase in water causes cell swelling and leads to necrosis of the cells, so called cytotoxic oedema. Cytotoxic oedema occurs rapidly as a result of disturbances in cell metabolism and ion homeostasis, without disruption of the BBB. Cytotoxic oedema is principally caused by the reduction in Na^+/K^+-ATPase activity. Such oedema occurs following both 60 and 120 min of transient occlusion and also following permanent MCA occlusion but is not seen after only 30 min occlusion (Yang *et al.*, 1992). Vasogenic oedema occurs at later time points following occlusion and results from the breakdown of the BBB with a net gain of interstitial fluid (Betz *et al.*, 1994; Samdani *et al.*, 1999). Swelling of the brain with no apparent disruption of the BBB suggests that cytotoxic oedema is primarily responsible, whereas at later time points, vasogenic oedema is the main contributor to damage.

Changes in BBB permeability have been demonstrated following an ischaemic insult (Belayev *et al.*, 1996a) and are likely to induce oedema formation that further exacerbates

tissue damage as BBB breakdown occurs faster than oedema formation (Hatashita & Hoff, 1990). In a global ischaemia experiment, Schwab *et al.* (1997) demonstrated that the regions that were most vulnerable to ischaemic damage corresponded to those areas with increased water content (Schwab *et al.*, 1997). BBB breakdown is accompanied by extravasion of blood borne agents that contribute to damage. If reperfusion occurs, the brain tissue is exposed to further potential mediators of damage (Kastrup *et al.*, 1999).

Biphasic opening of the BBB has been demonstrated following transient MCA occlusion (1 hr), occurring at 15 min after recirculation and again at 5 and 72 hrs (Kuroiwa *et al.*, 1985). Belayev *et al.* (1996) showed an initial acute disruption of the BBB using Evans Blue extravasion between 3 and 5 hrs again following transient MCA occlusion with a later more widespread increase in permeability at 48 hrs. Most prominent leakage of serum protein was observed in the striatum with leakage in the cortex at later time points (Belayev *et al.*, 1996a). In permanent MCA occlusion, there is no early disruption of the BBB as occurs in a transient occlusion (Betz *et al.*, 1994) suggesting the secondary disruption is a reperfusion related event. In the photothrombic model of MCA occlusion, BBB breakdown occurs more rapidly (Forsting *et al.*, 1994) perhaps reflecting damage to the endothelium by free radicals produced by the dye breakdown and thrombus formation (Dalton Dietrich, 1999). The comparison of two models of MCA occlusion (photothrombic and electrocoagulation) showed that oedema formation was greater in the photothrombic model despite similar damage in both (Takamatsu *et al.*, 1998).

Suggested mechanisms of passage of macromolecular substances across the BBB in pathological situations include acute destruction of the endothelium and opening of endothelial tight junctions as a consequence of disturbed autoregulation although it is a subject of much debate (Belayev *et al.*, 1996a). It is also possible that BBB damage occurs when a surge of blood returns to the ischaemic tissue. This may be an artefact of the

experimental model used. In the monofilament model, the introduction and withdrawal of the nylon suture may cause endothelial damage, at least in the internal carotid artery and therefore the method of occlusion may influence BBB patency (Gartshore *et al.*, 1997).

As with the extent of damage, the disruption of the BBB is believed to depend on the level of blood flow reduction and duration of the insult (Hallenbeck & Dutka, 1990). In global ischaemia, the insult is less severe and BBB integrity is maintained (Schwab *et al.*, 1997). Instantaneous massive breakdown of the BBB is seen following 3 hrs of MCA occlusion with 3 hrs of reperfusion also suggesting that late reperfusion causes more severe BBB disruption (Kastrup *et al.*, 1999). In patients fatal oedema formation has been reported following thrombolysis with tissue plasminogen activator (tPA) (Koudstaal *et al.*, 1995). Recently, Pfefferkorn *et al.* (2000) investigated plasminogen activation in focal cerebral ischaemia and reperfusion. They showed increases in plasminogen in the ipsilateral hemisphere and suggested that microvascular injury may be a consequence of the proteolytic functions of the plasminogen-plasmin system as plasmin directly degrades components of the extracellular matrix. Administration of tPA therefore would increase oedema formation and also haemorrhage as a result of compromised microvascular patency (Pfefferkorn *et al.*, 2000). Reperfusion therefore carries an inherent risk of vasogenic oedema and the integrity of the BBB may play an important role in during post-ischaemic reperfusion (Kastrup *et al.*, 1999). Experimentally, reperfusion exacerbates the swelling of the brain following ischaemia. Reperfusion provides hydrostatic pressure for water movement across the vessels within the ischaemic territory and additionally provides energy for the ATPases on the vessel walls that maintain the influx of Na^+ into the brain parenchyma (Gartshore *et al.*, 1997).

GLUTAMATE

Despite being principle excitatory neurotransmitter in the mammalian brain, glutamate is toxic to neurones (Obrenovitch *et al.*, 1996). Neurotoxicity caused by the application of exogenous glutamate, termed excitotoxicity, has been demonstrated and studied both *in vitro* systems (Choi *et al.*, 1988; Choi, 1990a) and in *in vivo* (Murata *et al.*, 2000; Wahl *et al.*, 1994). Increases in concentrations of EEAs, including glutamate, have been correlated with histopathological damage following an ischaemic insult (Butcher *et al.*, 1990). Additionally, compounds that antagonise NMDA (MK-801) and AMPA (NBQX, YM90K) glutamate receptors have been shown to be neuroprotective in a number of animal models of focal cerebral ischaemia indicating a central role for the neurotransmitter in the pathophysiology of the disease (Lipton, 1999).

Glutamate in neurones and glia of the brain is present principally in metabolic or neurotransmitter pools and its intracellular concentration is 4 times greater in than that in the extracellular environment (Obrenovitch & Richards, 1995). Normal extracellular glutamate levels measured by microdialysis are 1-5 μM (Lipton, 1999) with brief increases (1-2 mM) associated with synaptic transmission (Obrenovitch *et al.*, 2000). Under mild ischaemic conditions and *in vitro*, the concentration of EEAs can increase 2-5 fold and under severe ischaemic conditions between 10-60 fold (Martin *et al.*, 1994). Wahl *et al.* (1994) suggested that the majority of glutamate released into the extracellular space during severe ischaemia is of metabolic origin and from both neurones and glia (Wahl *et al.*, 1994). In both focal and global ischaemia, extracellular glutamate begins to increase rapidly, within 1-2 mins, of an insult (Lipton, 1999). A biphasic change in glutamate release following an insult confirms that there are several mechanisms of release and multiple sub-cellular origins (Obrenovitch & Richards, 1995; Szatkowski & Attwell, 1994).

The excessive release of intracellular glutamate from its stores following an ischaemic insult can occur through a number of pathways, either individually or in concert and can directly and indirectly regulate the downstream mechanisms of cell death (***Figure 5***) (Krupinski *et al.*, 2000). Initially, neuronal depolarisation causes the release of synaptic glutamate as would occur under normal conditions. Thereafter, the imbalance of ATP driven glutamate uptake and leakage of the transmitter from the cells, Ca^{2+} dependent exocytotic release for neurotransmitter pools, reversal of the glutamate transporters of neurones and/or glia and ultimately cell lysis causes a huge increase in extracellular glutamate concentration affecting neighbouring cells and creating a vicious circle of depolarisation and further neurotransmitter release (Kermer *et al.*, 1999; Rossi *et al.*, 2000). Ischaemic glutamate release is thought to be modulated by Na^{+} fluxes, free radicals/NO, temperature and some kinase pathways (Lipton, 1999). Released glutamate is rapidly cleared during reperfusion suggesting uptake mechanisms affected by ischaemia recover once membrane gradients are restored. The consequences of such an efflux of the transmitter has down stream effects that challenge cell viability. Obrenovitch & colleagues (2000) suggest that the glutamate alone is not the principle mediator of the cell damage following ischaemia but is one of the contributors (Obrenovitch *et al.*, 2000). The neurotransmitter activates both ion channel coupled receptors (NMDA, AMPA and kainate) and metabotropic receptors that are linked to second messenger cascades. Excessive activation of the ionotropic receptors leads to perturbations in ion homeostasis and massive Ca^{2+} influx principally through the NMDA receptor. A large increase in intracellular Ca^{2+} through ion channels leads to Ca^{2+} induced Ca^{2+} release from major intracellular stores such as the endoplasmic reticulum (ER) and mitochondria (Siesjo *et al.*, 1999).

CALCIUM

Intracellular and extracellular Ca^{2+} levels are approximately 0.1 and 1000 $\mu mol.l^{-1}$, respectively. Ca^{2+} fluxes, integral to signal transduction, are tightly regulated and Ca^{2+} homeostasis is maintained by ATP driven ion exchangers. The majority of the intracellular Ca^{2+} in the cytoplasm is bound to proteins or phospholipids or sequestered into the ER, mitochondria and calciosomes (Kristian & Siesjo, 1998; Siesjo *et al.*, 1999). Voltage-gated Ca^{2+} channels are located both pre- and post synaptically at excitatory synapses in the CNS where transmission is mediated by glutamate (Morley *et al.*, 1994).

The Ca^{2+} hypothesis was originally embraced to explain the link between excessive Ca^{2+} influx and myocardial damage (Dhalla *et al.*, 1999; Jennings *et al.*, 1960; Kristian & Siesjo, 1998) and was then implicated in neuronal death in a variety of neurodegenerative disorders (Kristian & Siesjo, 1998). The Ca^{2+} overload in the cytosol of ischaemic tissues occurs because of failure of ATP-dependent extrusion, influx of Ca^{2+} ions through voltage-dependent channels activated by membrane depolarisation and activation of glutamate receptors, principally NMDA receptors. Intracellular Ca^{2+} binding and sequestration is also affected and Ca^{2+} is displaced and released from intracellular locations such as the mitochondria and the ER (Mattson *et al.*, 2000b; Siesjo *et al.*, 1999). Lee *et al.* (1999) proposed that the survival of neuronal cells is critically dependent upon an optimal intracellular Ca^{2+} 'set point'. Inadequate Ca^{2+} places the cells at risk of apoptosis, intermediate Ca^{2+} levels where survival is favoured and elevated Ca^{2+} levels is toxic and leads to necrosis (Lee *et al.*, 1999). Following 2 hrs of MCA occlusion, Kristián *et al.* (1998) demonstrated that in the focal areas of the ischaemic brain virtually all the extracellular Ca^{2+} was translocated to the intracellular fluids. With recirculation, the extracellular Ca^{2+} concentration only returned to 50 % of the pre-ischaemia value for 6 hrs

and then decreased again. In the penumbral areas, there was a decrease in extracellular Ca^{2+} that returned to near normal levels upon recirculation proving that ischaemia has a profound effect on cell Ca^{2+} metabolism (Kristian *et al.*, 1998).

The massive increase of intracellular Ca^{2+} has a number of consequences. In addition to increasing the rate of ATP depletion as the cells attempt to maintain normal homeostasis, Ca^{2+} activates enzymes (protein kinase C, phospholipases, proteases, nitric oxide synthase) that cause intracellular damage, activate second messenger pathways that culminate in altered gene expression and increase the production of free radicals (Kermer *et al.*, 1999; Siesjo, 1993; Sweeney *et al.*, 1995). Many of these are energy consuming processes and so lead to further reductions in already critically compromised ATP levels.

ZINC

There is evidence to suggest that Ca^{2+} is not the only divalent cation that is toxic under ischaemic conditions (Lee *et al.*, 1999). Zinc (Zn^{2+}) is present in all cells as a component of metalloenzymes and transcription factors but also acts as a neurotransmitter or neuromodulator. Elevated levels of extracellular Zn^{2+} are known to be toxic to cultured neurones (Choi *et al.*, 1988). It has been suggested that excessive Zn^{2+} release and entry into cells via voltage gated channels (in a similar manner to Ca^{2+}) reaches levels that may alter the function of cellular macromolecules. Studies with chemical chelators of Zn^{2+} ions have been shown to reduce damage in global models of ischaemia. The protection afforded in these studies was not permanent suggesting that Zn^{2+} was involved in the rate of cell death but did not affect final outcome (Lee *et al.*, 1999). Studies in permanent focal ischaemia also suggest a link between Zn^{2+} release, translocation and brain injury (Sorensen *et al.*, 1998). It has also been suggested, from experiments *in vitro,* that Zn^{2+} may be neuroprotective by blocking the NMDA receptor (Choi, 1990b).

FREE RADICALS & NITRIC OXIDE

Under normal physiological conditions, potentially damaging free radicals (classified by virtue of their unpaired electrons) such as superoxide anions ($O_2^{\bullet-}$) and hydroxyradicals ($^{\bullet}OH$) together with hydrogen peroxide (H_2O_2) and NO are generated by mitochondrial respiration in complex I and III of the electron transport chain (Chan, 1999; Werns & Lucchesi, 1990; Kuroda & Siesjo, 1997). The generation of toxic free radicals and reactive oxygen species (ROS) by all aerobic cells is normally kept in check by both enzymatic and non-enzymatic defence systems. Cells contain vitamins such as ascorbic acid and α-tocopherol that act as free radical scavengers. They also contain enzymes such as superoxide dismutase, glutathione peroxidase and catalase that metabolise free radicals and their precursors (Siesjo, 1992b). Under ischaemic conditions, free radical production may outstrip the capabilities of the endogenous defence mechanisms and the activity of protective enzymes may be compromised by energy depletion. When reperfusion is established following an ischaemic insult, the return of blood to the compromised tissue leads second burst of free radicals as substrates for oxidation are provided and partial recovery of the bioenergetic state of the tissue occurs (Chan, 1996; Kuroda & Siesjo, 1997).

The cellular source of free radicals is difficult to establish because of their short life span *in vivo*. Both neurones and glia can generate free radicals and it is thought that the endothelium and activated leukocytes, particularly during reperfusion, are candidates for free radical production (Matsuyama, 1997). The link between free radicals and cell damage in ischaemia is strengthened by observations that free radical scavengers such as allopurinol and methylthiourea reduce infarct size following permanent MCA occlusion (Siesjo, 1992b). The radical spin trap α-phenyl-*N*-*tert*-butyl nitrone (PBN) also ameliorated damage following

2 hrs of MCA occlusion (Kristian & Siesjo, 1997). Superoxide dismutase over-expressing and knockout mice show protection against and exacerbation of damage, respectively, after an ischaemic insult (Chan, 1999). The formation of superoxide, hydrogen peroxide, peroxynitrite and hydroxyl radicals leads to peroxidation of lipid membranes, causes damage to proteins and DNA and thereby compromising the structure and functioning integrity of the cells (Dawson, 1994).

NO is an important physiological regulator of functions such as vasodilation, neurotransmission and under physiological conditions is found at low levels in the body (Dawson, 1994; Dawson *et al.*, 1999). Levels of nitric oxide are controlled by constitutively expressed neuronal and endothelial isoforms of nitric oxide synthase (nNOS/NOS-I and eNOS/NOS-III, respectively). During ischaemia-reperfusion and other neurodegenerative processes in the CNS, the concentration of NO is rapidly increased by hyperactivation of nNOS. An unnaturally high level of NO in the brain is detrimental as its reaction with superoxide anions produces toxic compounds such as peroxynitrite and hydroxyl radicals. High levels of NO are produced under pathological conditions such as inflammation by an inducible form of NO synthase (iNOS/NOS-II) supporting a beneficial role of NO production where its expression leads to the elimination of the inflammatory agents (Colasanti & Suzuki, 2000).

The role of NO is cerebral ischaemia is complex and unclear as it has been suggested to be both cytotoxic and cytoprotective (Lipton & Wang, 1996; Anderson & Meyer, 2000; Kermer *et al.*, 1999; Mason *et al.*, 2000). NOS inhibitors have been shown to be both protective against cerebral infarction (Buisson *et al.*, 1992) and also to increase post-ischaemic damage (Dawson, 1994). NO precursors such as L-arginine and nitrovasodilators have been shown to have no effect on lesion size (Anderson & Meyer, 2000) but have also been shown to reduce damage (Zhang *et al.*, 1997). NO donor compounds have been shown to be protective *in*

vitro and *in vivo* (Mason *et al.*, 2000). Additionally, ischaemic damage has been shown to be significantly reduced in neuronal NOS knockout mice (Chan, 1996). Possible explanations for the ambiguous nature of NO may be attributed to its vasodilatory properties (causing improvement of blood flow) or its inhibitory actions on the NMDA receptor (attenuating of Ca^{2+} influx) that may be a consequence of its redox state (Lipton & Wang, 1996) and the complexity of the regulatory mechanisms of the nitric oxide synthases that control NO production (Colasanti & Suzuki, 2000). Anderson *et al.* (2000) have proposed that the severity of the ischaemia may explain the relative importance of NO in the ischaemic tissue. More severe ischaemic insults are linked with greater tissue acidosis related to the anaerobic metabolism that inhibits the NOS activity (Anderson & Meyer, 2000) .

The point at which NO is produced following an insult may be of great importance and explain the disparate findings regarding its role in cerebral ischaemia. In the early stages of local blood flow reduction, eNOS activity may be beneficial producing NO that causes vasodilation. Later, when the vascular effects are no longer protective, nNOS activity produces NO that is damaging to the tissue. At an even later time point the long lasting production of NO by iNOS contributes to the progression of cell damage (Kermer *et al.*, 1999). These ideas regarding the temporal production of NO are supported by a series of *in vitro* studies with NOS inhibitors where early inhibition produced increased damage whereas delayed inhibition had either no effect or only a slightly beneficial impact on infarct volume (Mason *et al.*, 2000). Disappointing neuroprotection achieved with NOS inhibitors may be due to the fact that inhibition of NO formation only leaves other free radicals that cause cell damage (Dawson, 1999). This idea is supported by the observation that rats treated with a combination of NOS inhibitor and free radical scavenger have a greater reduction in infarct volume (Spinnewyn *et al.*, 1999).

MITOCHONDRIAL DYSFUNCTION

Stroke can be described essentially as a disease of mitochondrial dysfunction. Mitochondria play a central role in both apoptosis and necrosis and are therefore key players in the evolution of ischaemic damage (Halestrap *et al.*, 2000). The mitochondria are strongly linked to glutamate excitotoxicity, which is central to the pathophysiology of ischaemic cell death (Stout *et al.*, 1998). Oxidative phosphorylation in the mitochondria requires an intact inner membrane that is impermeable to all but a few selective metabolites and ions. If the membrane potential and integrity is compromised or lost, the organelles become uncoupled and hydrolyse ATP rather than synthesising it (Halestrap *et al.*, 2000).

There are at least three general mechanisms (with inter-related effects) that are known by which the mitochondria participate in cell death: disruption of oxidative phosphorylation and ATP production, release of proteins that trigger caspase activation and the alteration of the redox potential of the cell (Green & Reed, 1998). Following transient ischaemia, there appear to be two components to mitochondrial dysfunction. The first component is an initial decline in respiratory function at the onset of ischaemia. Secondary deterioration in mitochondrial function is associated with reperfusion when there is partial recovery in ATP levels (Nakai *et al.*, 1997). During ischaemia, the accumulation of Ca^{2+} in the mitochondria can trigger the assembly and opening of a high conductance pore, the mitochondrial permeability transition pore (MPT), in the inner membrane of the mitochondria. The pore is thought to be formed by a cyclophilin-D mediated conformational change in the adenine nucleotide translocase proteins in the membrane (Halestrap *et al.*, 2000). The formation of this pore leads to the collapse of the electrical potential, increased acidosis, cessation of ATP production and generation of free radicals. The pore also allows the release of Ca^{2+} and Mg^{2+}, as well as low and high molecular weight compounds (Kristian & Siesjo, 1998). Transient opening of the MTP causes halting of ATP synthesis, swelling of the organelle,

rupture of the outer membrane and release of cytochrome *c* (an essential component of the respiratory electron transport chain) and subsequent activation of caspases that can set the apoptotic cascade in motion. The closure of the pore allows restoration of ATP production and thus leads to cell death by apoptosis as opposed to by necrosis (Halestrap *et al.*, 2000).

Cyclosporin A (CsA), which has been shown to neuroprotective in a number of models and species of cerebral ischaemia, is thought in part to be neuroprotective as it blocks the formation of the MTP (Sharkey *et al.*, 2000). CsA prevents MPT formation by blocking the translocation of the mitochondrial matrix specific cyclophilin-D (small intracellular regulatory protein of the immunophilin family) to the inner membrane of the mitochondria and thereby decreasing sensitivity to Ca^{2+} ions (Friberg *et al.*, 1998).

As mentioned previously, the mitochondria are thought to be involved in glutamate toxicity (Ankarcrona *et al.*, 1995). The mitochondria have a large capacity for Ca^{2+} and may be neuroprotective by removing the Ca^{2+} ions from the cytoplasm. However, the uptake of Ca^{2+} may be detrimental to the mitochondria as the excessive glutamate stimulation and activation of intracellular enzymes can cause the generation of free radicals. Free radicals can potentially damage structures of the cells including the mitochondria and thus confound the energetic crisis of the cells (Stout *et al.*, 1998).

GENE EXPRESSION & PROTEIN SYNTHESIS

Focal ischaemia causes changes in gene expression within minutes of the onset of ischaemia, particularly in the ischaemic hemisphere (Johansson *et al.*, 2000). Most of the genes that respond rapidly are members of the immediate early gene (IEG) family such as c-*fos* and c-*jun* those products are transcription factors and regulate the expression of other genes (An *et al.*, 1993; Soriano *et al.*, 2000). Under normal conditions in the brain, the expression of the

IEGs is low. The stimulation of cells that causes changes in the cytoplasm such as elevated Ca^{2+} levels or the activation of kinases (in particular, protein kinase A or C) results in phosphorylation of deoxyribonucleic acid (DNA) binding proteins that bind to response elements that regulate gene transcription. The products of the IEGs regulate the expression of downstream effector genes that affect the functional status and viability of the cells (Akins *et al.*, 1996). Evidence is accumulating that suggests that signalling pathways leading to gene expression can intersect and can alter the cell's response to a given stimulus. IEGs dimerise with different transcription factors with different outcomes (Kermer *et al.*, 1999).

The genes induced following a transient and permanent MCA occlusion may differ because of the differences in biochemical events and every gene may vary spatially and temporally with varying degrees of ischaemia. Following a mild focal ischaemic insult, IEG expression is limited to the ischaemic zone whereas more severe insults tend to induce expression in regions beyond this (An *et al.*, 1993; Collaco-Moraes *et al.*, 1994). Gene expression may also differ in different models of occlusion. For example, genes induced after transient ischaemia may reflect the prominent role of free radicals and oxidative stress associated with reperfusion whereas the same genes may play a less important role in permanent arterial occlusion (O'Neill & Kaltschmidt, 1997; Sharp *et al.*, 2000). Other genes that are induced following ischaemia including heat shock proteins (Sharp & Sagar, 1994), neurotransmitters and corresponding receptors (Kogure & Kato, 1993), transcription regulators such as nuclear factor of kappa light chain gene enhancer in B cells (NF-κB) (Salminen *et al.*, 1995) and neurotrophic factors including nerve growth factor (NGF) (Mattson, 1997).

The expression of the transcription regulator NF-κB has also been of much interest in the stroke research field in the past few years (Carroll *et al.*, 1998; Carroll *et al.*, 2000; Christman *et al.*, 2000; Gabrielet al.1999; Schneider *et al.*, 1999; Stephenson *et al.*, 2000)

and is of particular interest in the context of an inflammatory response as it is an immediate early transcriptional activator of the genes encoding inflammatory cytokines (Zwacka *et al.*, 1998). NF-κB is a pre-formed factor that is usually sequestered in the cytoplasm of cells by inhibitor of kappa light chain gene enhancer in B cells (IκB), which when phosphorylated and/or cleaved frees the bound NF-κ allowing translocation to the nucleus. In the nucleus, NF-κB influences the expression of a wide variety of genes including many stress activated genes (MacManus & Linnik, 1997). Again, as with other members of the pathophysiological cascade of ischaemia such as NO, NF-κB can be either detrimental or beneficial to cell survival and may be highly context dependent (Kristian & Siesjo, 1997; Sharp *et al.*, 2000).

The induction of heat shock proteins occurs within the same time frame as the expression of the IEGs and may be neuroprotective (Akins *et al.*, 1996). These proteins along with other chaperone proteins aid the restoration of structure and function of denatured proteins. Following ischaemia, heat shock protein gene expression is seen in glial cells and in cells in the penumbra (Sharp *et al.*, 1999). There is also an alteration in expression of genes involved in the apoptotic death cascade such as bcl-2, Bax and the caspases (*see Neuronal Death: Necrosis & Apoptosis*). An increase in nitric oxide synthase mRNAs is seen in the initial hours following cerebral ischaemia and is different depending on occlusion type (MacManus & Linnik, 1997). Another area that has been intensively investigated is the expression of genes of the inflammatory cascade such as cell adhesion molecule as and cytokines (*discussed later in this chapter and in Chapter 6*).

Although mRNA is frequently studied, it is critical to study protein function. If a gene is involved in ischaemic pathology, the associated protein must be present (Sharp *et al.*, 2000). Genes such as the IEGs are detected in both core and penumbral regions following focal ischaemia but expression of proteins may differ as protein synthesis may be affected in

regions of dense ischaemia (Akins *et al.*, 1996). Protein synthesis is a prerequisite for ong-term survival of cells and is extremely sensitive to cell energy charge and ionic content (Lipton, 1999). Prolonged inhibition of protein synthesis, if uncompensated, will ultimately lead to cell death. Protein synthesis is an energy requiring process and thus depletion of ATP during ischaemia is responsible for its cessation. During reperfusion synthesis may still be compromised despite the return of energy yielding compounds (Krause & Tiffany, 1993). In a study in mice, Hara *et al.* (2000) showed that 1 hr of reperfusion led to full recovery of energy metabolism but not protein synthesis (Hara *et al.*, 2000). Protein synthesis is dependent not only on intact DNA and intact transcription machinery but also intact transport machinery for transcription factors and messenger RNA (mRNA) and intact translation machinery (Krause & Tiffany, 1993). Soriano *et al.* (2000) propose that the inhibition of protein synthesis after focal ischaemia is not caused by inhibition of transcription but by inhibition at the translation step (Soriano *et al.*, 2000). Free radicals and increased Ca^{2+} levels following interruption of cerebral blood flow can potentially damage all the components of the protein synthesis cascade. There is little or no protein synthesis in the core of an ischaemic lesion as this region is ATP depleted but synthesis does continue in the cells in the penumbral zones that will survive the insult (Sharp *et al.*, 2000).

NEURONAL DEATH: NECROSIS & APOPTOSIS

There are two principal death pathways, necrosis or a mechanism resembling apoptosis that occur following an ischaemic insult in any organ or tissue. Apoptosis was defined by Kerr *et al.* in 1972 and was originally based on morphological differences in thymocytes that appeared to die by two processes (Kerr *et al.*, 1972). Necrosis appears to be a passive mechanism of cell death. This cell death is associated with derangement of ion homeostasis related to membrane failure and is characterised by early cell and organelle (mitochondrial)

swelling. This is followed by loss of cytoplasmic membrane integrity and cellular disintegration, often accompanied by an inflammatory response (Snider *et al.*, 1999) and usually affecting a large group of adjacent cells (Banasiak *et al.*, 2000). Apoptosis or apoptosis-like cell death is viewed as an energy requiring 'programmed' physiological response that is most apparent during development where more cells than are ultimately required die. In this respect, apoptotic cell death sculpts, shape and optimises function (Golstein, 1998). During development apoptosis is heavily influenced by the availability of growth factors and is not confined to neurones as it also occurs in glial cells (Kuschinsky & Gillardon, 2000). Apoptosis also plays a role in rapidly dividing tissue such as intestinal villi and dysfunction of the death pathways may be linked to tumour growth (Kinloch *et al.*, 1999). Apoptotic cell death plays an important role in the removal of compromised cells without the induction of a major inflammatory response (Linnik & Ringer, 1999).

It has been recently suggested that the 'classical' concept of cell death following ischaemia may not be completely accurate (Kuschinsky & Gillardon, 2000; Lee *et al.*, 2000). Although there are distinct morphological characteristics associated with the different pathways it is likely that necrosis and apoptosis are part of a continuum of cell death and that similar mechanisms may be operative in both processes (Roy & Sapolsky, 1999). There is also a third potential form of cell death, autophagocytotic cell death that has been described and may occur in ischaemia although it has not been well investigated. Autophagocytosis is the mechanism by which protein turnover occurs in normally functioning cells and which if activated under pathological conditions, may lead to destruction of cells (Lipton, 1999). The execution of cell death following an insult may be the sum total of a number of complex pathways that interact that may be dictated by the metabolic status of the cells (Nicotera *et al.*, 1999b).

Necrotic cells exhibit pyknosis (shrinkage) and eosinophila and as stated before, are grossly distinguished by loss of plasma membrane integrity. Ultrastructural changes include clumping of heterochromatin and swelling of endoplasmic reticulum cisternae (Garcia *et al.*, 1995). The DNA in necrosising cells also undergoes random fragmentation following histone proteolysis (Banasiak *et al.*, 2000). Apoptosing cells exhibit shrinkage of cytoplasm, condensation of nuclear material into 'clumps', nuclear condensation and fusion of the ER with the plasma membrane. It is this formation of membrane bound apoptotic bodies that contain cytoplasmic organelles and nuclear fragments which gives the cells a 'blebbed' appearance (Banasiak *et al.*, 2000). Analysis of the DNA fragments from ischaemic tissue reveal that staggered ends as opposed to the blunt DNA ends of cells undergoing developmental apoptosis supporting the notion of an non-classical apoptotic cell death following ischaemia (Kuschinsky & Gillardon, 2000). Nuclear DNA damage following cerebral ischaemia may involve two distinct mechanisms: oxidative injury and endonuclease-mediated DNA fragmentation (Charriaut-Marlangue *et al.*, 1995; Chopp, *et al.*, 1996). In a permanent occlusion model, Nagayama *et al.* (2000) demonstrated that in the penumbral zone of a cerebral insult that oxidative injury to nuclear DNA (direct damage from reactive oxygen species) may be a significant and contributory cause to the expansion of a brain damage and could perhaps be an early, potentially reversible event. Endonuclease-mediated fragmentation is a hallmark of apoptosis and occurs relatively late following ischaemia (Nagayama *et al.*, 2000).

The contribution of apoptosis in ischaemia is a controversial subject although there is evidence to suggest that key components of the cell death programmes contribute cell death in experimental animals (Banasiak *et al.*, 2000; Du, *et al*, 1996; MacManus & Linnik, 1997; Nicotera *et al.*, 1999b; Rabuffetti *et al.*, 2000; van Lookeren & Gill, 1996). A few studies suggest that apoptosis occurs in stroke patients however such studies are limited by the difficulty in obtaining post-mortem tissue at time points that correspond to the time window

when cell death is occurring (Mattson *et al.*, 2000a). Observations from animal stroke models, suggest that apoptosis occurs in the in penumbral regions of the infarct and while necrosis predominates in the more severely stressed ischaemic core (Akins *et al.*, 1996; Charriaut-Marlangue *et al.*, 1996; Linnik & Ringer, 1999). Apoptosis in the penumbra may be the consequence of milder stresses than those experienced in the core but may also be due to secondary mediators such as free radicals, glutamate, cytokines and lipid peroxidation products resulting from damage to the tissue in the core (Nicotera *et al.*, 2000). Li *et al.* (1995) reported that, following transient MCA occlusion, apoptotic cells (terminal deoxynucleotidyl transferase nick end labelling (TUNEL) stained) were primarily localised to the boundary zones of the infarct and also concluded that apoptosis accompanies necrosis (Li *et al.*, 1995a). Du *et al.* (1996) suggested that neuronal apoptosis was present after mild focal ischaemia and that the lesion developed at a later time point (14 days) although this has been difficult to replicate. Following a severe insult, there was only limited evidence of apoptosis (Du *et al.*, 1996).

The two main forms of cell death may therefore not be mutually exclusive and overlap both spatially and temporarily but it is thought that necrotic mechanisms occur earlier than apoptotic ones (Roy & Sapolsky, 1999; Velier *et al.*, 1999). It is also thought that moderately damaged cells die in the suicidal apoptotic manner once their repair capacity is exhausted and cells subjected to more severe insults die rapidly by necrosis (Kuschinsky & Gillardon, 2000). Residual mitochondrial function (ATP synthesis) is required for apoptosis and it is important to remember that ATP depletion is central to the pathology of cerebral ischaemia (Siesjo, 1984; Siesjo, 1992a). This is highlighted by experiments where the reduction of ATP levels in cells that have received an apoptotic stimulus causes necrosis instead of the expected apoptosis (Nicotera *et al.*, 1999b). The relative contributions of each type of cell death in ischaemia is difficult to assess as both electrophoresis laddering patterns of genomic DNA and TUNEL staining can be inconclusive and are best supported by

electronmicroscopy and discrimination of morphological features (Banasiak *et al.*, 2000; Bortner *et al.*, 1995).

The fundamental principles of the apoptotic cascade have been established by studies on the nematode *Caenorhabditis elegans* (*C. elegans*) because of the homology in structure and function of its death genes compared with vertebrates (Kuschinsky & Gillardon, 2000). It is the interplay of pro- and anti-apoptotic genes that determines cell survival. From mutant studies in *C. elegans*, *ced-3* and *ced-4* have been identified as essential for death (Ashkenazi & Dixit, 1998; Ellis *et al.*, 1991) whilst *ced-9* functions to protect cells from dying (Hengartner & Horvitz, 1994). In mammals, the homologous genes to *ced-3* and *ced-9* have been identified as interleukin 1β-converting enzyme (ICE) and bcl-2 respectively. ICE belongs to a family of death related genes termed cysteine aspartases (caspases) and is also known as caspase 1. The human homologue of *ced-4* has been identified as APAF-1, an activator of the caspase enzyme family (Hengartner, 1998).

The bcl-2 family consists of proteins with pro- and anti-apoptotic activity that are essential components of the death machinery in neurones (Adams & Cory, 1998; Antonsson & Martinou, 2000; Bergeron & Yuan, 1998; Chen *et al.*, 1995). Bcl-2 has been shown to inhibit apoptosis and has the capacity to promote neuronal survival in a number of experimental settings (Chen *et al.*, 2000). In ischaemia, bcl-2 appears to prevent cell death. This has been demonstrated using both transgenic and viral vector technology where infarct volume was decreased (Linnik & Ringer, 1999). Bcl-2 and other closely related genes (Bcl-x) interact with each other to regulate their function. Bcl-2 associated protein X (Bax) is a potent accelerator of apoptosis, which like bcl-2 is activated following ischaemia (MacManus & Linnik, 1997). Bcl-2 is found on the membranes of the ER, the nuclear envelope and the outer mitochondrial membrane and may play a role in the mitochondrial transition pore.

Other mechanisms that have been proposed for bcl-2, which may be important in ischaemia include the prevention of free radical production, modulation of intracellular Ca^{2+} or prevention of intracellular acidification (Banasiak *et al.*, 2000).

Caspases are proteases that are the main effectors in apoptotic cell death and to date more than 14 mammalian members of the family have been identified (Nicotera *et al.*, 2000b). They are expressed as inactive pro-enzymes (30-50 kDa) and are activated by proteolytic cleavage (Thornberry & Lazebnik, 1998, Nicholson & Thornberry, 1997). All caspases contain a conserved pentapeptide QACXG and are divided into three groups based on their tetrapeptide recognition sequences. The DexDases and (IVL)ExDases participate in the signalling and execution of apoptosis whereas the WEHDases are involved in inflammatory processes (Nicotera *et al.*, 2000). The cleavage of caspase pro-enzymes at specific sites is the trigger for cellular destruction cascade and can be initiated by activation of death ligand receptors (Fas/CD95) or by activated upstream caspases (Hengartner, 1998). The caspases appear to contribute to apoptosis through the direct disassembly of cell structures (Thornberry & Lazebnik, 1998). It has recently been proposed that the degree of caspase activation may be of importance suggesting that a moderate degree of caspase activation may not always result in cellular demise (Nicotera *et al.*, 2000). The degree of caspase activation may reflect the severity of the ischaemic insult (Matsushita *et al.*, 1998). In an ischaemic insult necrosis and apoptosis are present in adjacent cells suggesting a common extracellular environment and that the pathways leading to the different endpoints may overlap (Li *et al.*, 1997).

Caspase 1 expression is increased following ischaemia although in a delayed fashion and has been localised to the microglia (Bhat *et al.*, 1996). Studies with transgenic animals revealed that inhibition of caspase 1 decreased infarct size and improved neurological outcome (Friedlander *et al.*, 1996). The beneficial effects of caspase 1 inhibition are thought to be

mediated by the reduction in production of interleukin-1β (IL-1β) and although not obligatory during apoptosis it may be a contributing factor to cell death in ischaemia (Linnik & Ringer, 1999). The protective effects seen may be due to anti-inflammatory effects of reduction of IL-1β production, rather than inhibition of apoptosis or a combination of both (Loddick *et al.*, 1996; Schulz *et al.*, 1999). The inhibition of caspase 1 like enzymes pharmacologically using fluoromethyl ketone inhibitors (e.g. z-VAD.fmk) also causes a reduction in ischaemic damage (Wiessner *et al.*, 2000). Again it is difficult to distinguish anti-apoptotic and anti-inflammatory actions of these agents (Dalkara & Moskowitz, 1996; Rabuffetti *et al.*, 2000). In addition, inhibition of a specific member of the caspase family pharmacologically is questionable as the compounds may not be truly selective and may have other non-specific effects within cells (Nicotera *et al.*, 2000a).

Cerebral ischaemia & inflammation

In the past, the CNS was thought to be an immune privileged site because of the lack of expression of molecules involved in a classical immune response (Becher *et al.*, 2000). The brain has no lymphatic drainage, was previously assumed to have no classical antigen presenting cells and additionally because of the BBB, access of blood-borne immune cells is highly restricted (Perry *et al.*, 1993). Compared with other organs of the body, the immune reactions in the brain are markedly reduced (Becher *et al.*, 2000; Gebicke-Haerter *et al.*, 1996; Perry *et al.*, 1993). In recent years, the idea of immune privilege status of the brain has been re-examined and reviewed (Barone & Feuerstein, 1999; Hallenbeck, 1997; Jean *et al.*, 1998). The inflammatory processes of the brain are thought to be more subtle and differ from those in non-CNS tissue (Matyszak, 1998).

Inflammation following an ischaemic insult is believed to develop as a consequence of two sequential but closely linked processes: activation of the resident inflammatory cells of the brain and the mobilisation and infiltration of peripheral inflammatory cells (Stanimirovic & Satoh, 2000). In effect, the inflammatory response can be divided into endogenous and exogenous components. The development of a post-ischaemic inflammatory response is co-ordinated by the activation, expression and secretion of numerous pro-inflammatory genes and mediators by both parenchymal and endothelial cells. The initiation of the exogenous inflammatory response following an ischaemic insult takes place in the microvasculature as it provides an interface for circulating inflammatory cells such as the polymorphonuclear leukocytes (PMNLs) to adhere to before passing into the underlying parenchyma (del Zoppo *et al.*, 2000).

Inflammation in the brain following injury has been termed 'a dual edge sword' (Barone & Feuerstein, 1999). Classically, inflammation is the body's response to damage and is beneficial in terms of removal of compromised tissue and elimination of invading pathogens (Andersson *et al.*, 1992). However, in functioning to eliminate foreign entities, the response may in turn cause more damage to surrounding tissue and therefore have a detrimental outcome. There is also a regenerative component to inflammation, particularly in the CNS, were signalling molecules originating from cells associated with the inflammatory response promote cell survival and repair (Andersson *et al.*, 1992). The inflammatory response can therefore be viewed to be finely balanced with the potential to have devastating consequences under certain conditions (Becher *et al.*, 2000).

LEUKOCYTES & CELL ADHESION MOLECULES IN ISCHAEMIA

The role of white blood cells, particularly PMNLs and monocytes, in the pathophysiology of ischaemic disease has been studies for over 3 decades in organs such as the heart, intestine and skeletal muscle and have been implicated in the evolution of ischaemia-reperfusion injury (Hartl *et al.*, 1996; Vasthare *et al.*, 1990). The term PMNL refers to three of leukocytes types (eosinophils, basophils and neutrophils) found in the blood that have multi-lobed nuclei and abundant membrane-bound granules. The neutrophils are the most abundant type of leukocyte. The fourth type of leukocyte is the monocyte which is larger than the other types and has an oval or horseshoe-shaped nucleus and few cytoplasmic granules (Vander *et al.*, 1994). The leukocytes, as fundamental participants in an immune response of the body, are there as a defence to protect tissues but may also have deleterious effects in ischaemic brain tissue (Dallegri & Ottonello, 1997; Kubes & Ward, 2000). The accumulation of leukocytes and monocytes following both clinical and experimental cerebral ischaemia was first identified in the 1970s by Sörnäs *et al.* (1972) and Garcia *et al.* (1974) (Garcia & Kamijyo, 1974; Sornas *et al.*, 1972). These studies were followed by epidemiological studies in the 1980s, that suggested leukocytes contributed to the initiation of stroke by affecting blood dynamics or by participation in clot formation (Prentice *et al.*, 1982). In the late 1980s, the contribution of leukocytes in the pathogenesis of brain injury was intensively investigated by many groups and has been followed by a large number of studies that have assessed the role of these cells in cerebral ischaemia (Rothlein, 1997). Accumulated evidence indicates that leukocytes play a key role leading to secondary brain damage particularly during reperfusion (Jean *et al.*, 1998).

Leukocyte presence at the site of inflammation is dependent on the co-ordinated expression of cell adhesion molecules, both ligands and receptors on the invading cells and the vascular endothelium, respectively. These molecules orchestrate and facilitate the adherence of the

immune cells that is followed by the migration into the target tissue. There are three families of cell adhesion molecules including the integrins, the selectins and the immunoglobulin super family (Arvin *et al.*, 1996; Kochanek & Hallenbeck, 1992). Integrins are essential determinants in the adhesion of leukocytes to the endothelium and consist of a CD11 and a CD18 subunit. The most important integrins are LFA-1 and Mac-1 that both contain a common CD18 subunit (Kochanek & Hallenbeck, 1992). The integrins bind to members of the largest family of the cell adhesion molecules the immunoglobulin superfamily or the intercellular cell adhesion molecules (ICAMs) that includes intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1). These molecules are expressed on the endothelium and are modulated by inflammatory mediators such as the cytokines (Stanimirovic & Satoh, 2000; del Zoppo *et al.*, 2000). The third class of cell adhesion molecules is the selectins including E- and P-selectin that are expressed on activated endothelium and L-selectin that is constitutively expressed on leukocytes (Ley, 1996).

There is multistep recruitment (rolling-activation-adhesion) of leukocytes as they enter the site of inflammation. The initial interactions rely on the selectins that facilitate the transient and reversible adhesion known as rolling. P-selectin (pre-formed in the cell) is rapidly secreted from storage granules called Wiebel-Palade bodies (within the first 90 min following insult (del Zoppo *et al.*, 2000)) followed by transcription induction of E-selectin (4-6 hrs), cell adhesion molecules and integrins (Hallenbeck, 1996; Stanimirovic & Satoh, 2000). Firm adhesion occurs when inflammatory mediators released by the endothelial cells activate PMNLs and cause expression of integrins (LFA-1 and Mac-1) that bind to the ICAM receptors expressed on the endothelium. Transmigration then follows mediated by adhesion molecule interaction with the extracellular matrix (ECM) and the PMNLs enter the tissue (Ley, 1996).

In stroke patients, neutrophils have been detected within the first 18 hrs after an acute ischaemic stroke (Bednar *et al.*, 1991). Subsequent studies observed an upregulation in cell adhesion molecules indicating neutrophil activation in stroke patients as early as 4 hrs (Fassbender *et al.*, 1995). Patients who have suffered an acute ischaemic insult have a functional alteration in their systemic PMNLs where the cells are more susceptibly to activation and which could indicate a trend towards increased accumulation in infarcted tissue (Caimi *et al.*, 2000). An increased number of PMNLs have been found in the microvessels and ischaemic parenchyma of various species following cerebral ischaemia (Barone *et al.*, 1991; Barone *et al.*, 1992; Clark *et al.*, 1993; del Zoppo *et al.*, 1991; Garcia & Kamijyo, 1974; Hallenbeck *et al.*, 1986; Kochanek & Hallenbeck, 1992). In the first studies of leukocyte accumulation using a myeloperoxidase assay, PMNL accumulation was seen in the brain tissue at the periphery of the lesion with maximum infiltration at 48-72 hrs following permanent occlusion of the MCA. PMNLS were seen in the core of the infarct at 7 days (Garcia & Kamijyo, 1974). More recent studies have shown that the PMNLs are the first of the exogenous inflammatory cells to reach the cerebral parenchyma accumulating in the vasculature 1-4 hrs post recirculation and peak in the parenchyma between 24 and 72 hrs (Garcia *et al.*, 1994; Hallenbeck, 1996). In rats subjected to permanent MCA occlusion, leukocytes are detected in the vessels of as early as 30 min post-occlusion (Garcia *et al.*, 1994). Again in a rat model of focal cerebral ischaemia it has been shown that neutrophil infiltration occurs earlier and to a greater extent in reperfused tissue (Clark *et al.*, 1994a). Zhang *et al.* (1994) correlated maximum infarct expansion with the time of neutrophil infiltration (6-24 hrs) following transient MCA occlusion (Zhang *et al.*, 1994).

It has been proposed that PMNLs obstruct the cerebral vessels and contribute to the 'no-reflow' phenomenon (Mori *et al.*, 1992). The no-reflow phenomenon has been demonstrated in the baboon (del Zoppo *et al.*, 1991) although as a phenomenon it has been questioned (Aspey *et al.*, 1989). Leukocytes may have other detrimental effects such as the

release of vasoactive mediators, cause alteration of the vasoreactivity of the cerebral vessels and release of cytotoxic enzymes and free radicals that can threaten the brain tissue (Pantoni *et al.*, 1998). Data that implicates leukocytes and other white blood cells such as monocytes in the pathology of cerebral ischaemia comes from studies that block or alter the function of these cells. Animals that have been rendered neutropenic by anti-neutrophil serum or by chemical means (vinblastine) have smaller infarcts than non-treated animals. These results are interpreted cautiously as the rheological effect of the treatments may influence outcome and in some cases development of damage was only delayed and had no effect on final lesion size (Bednar *et al.*, 1991). More convincing results have been obtained in experiments where antibodies that reduced circulating leukocytes were administered prior to occlusion and caused reduction in lesion volume (Matsuo *et al.*, 1994; Shiga *et al.*, 1991). Experimental strategies using antibodies directed at the cell adhesion molecules (anti-ICAM-1 or anti-CD11b antibodies) have proved successful in experimental models in terms of damage reduction although there is somewhat confusing data regarding this therapeutic approach from both pre-clinical investigations and clinical trials. Takeshima *et al.* (1992) demonstrated that an anti-CD11b/CD18 antibody did not afford protection from severe focal cerebral ischaemia in the cat although neuroprotection was assessed only 4.5 hrs post ischaemia using 2,3,5-triphenyltetrazolium chloride (TTC) staining. It is questionable if the damage or any neuroprotection would be clearly evident at this time point (Takeshima *et al.*, 1992). Zhang *et al.* (1995) reported no reduction in lesion volume with either anti-ICAM-1 or anti-CD11b antibodies in a permanent model of MCA occlusion in the rat (Zhang *et al.*, 1995). However, in the transient model, PMNL infiltration and lesion volume were reduced even if treatment was administered 2 hrs post-occlusion (Zhang *et al.*, 1995). Chen *et al.* (1994) showed similar results and correlated the reduction in PMNL accumulation with the reduction in damage (Chen *et al.*, 1994). Treatment with the anti-CD11b antibodies did not delay the maturation of the lesion, as the reduction in ischaemic damage was still evident at 7 days post-treatment (Zhang *et al.*, 1995). In contrast to the anti-ischaemic effectiveness of the

anti-adhesion molecule strategies in animal models, the trial for the murine anti-ICAM antibody for stroke (Enlimomab trial) failed, demonstrating the difficulties of extrapolation of animal data to the clinic (del Zoppo *et al.*, 2000). However, this apparent failure maybe due to the lack of reperfusion in the patients and perhaps positive results would have been seen had tPA been included in the treatment regime.

Studies in baboons and in rats subjected to MCA occlusion have shown the upregulation of selectin molecules on the microvascular endothelium (Okada *et al.*, 1994; Wang *et al.*, 1994b). ICAM-1 mRNA and protein levels are increased in focal cerebral ischaemia and have been correlated with the infiltration of neutrophils in a variety of studies (Winquist & Kerr, 1997; Matsuo *et al.*, 1994; Shiga *et al.*, 1991; Wang *et al.*, 1994b; Wang & Feuerstein, 1995). ICAM-1 expression is strongly expressed on ipsilateral cerebral vessels when compared with the contralateral cortex (Schroeter *et al.*, 1994). Selectins are expressed within the first few hours following an embolic stroke in the rat, peaking between 6 and 12 hrs. In contrast, the ICAMs are maximal at 48 hrs (Zhang *et al.*, 1999b). In a study using spontaneously hypertensive rats subjected to permanent or transient MCA occlusion, Morikawa *et al.* (1996) showed differences in blocking selectin binding in the two occlusions similar to that observed with the anti-CD11b antibodies i.e. effective in the transient model of occlusion but not the permanent model (Morikawa *et al.*, 1996). Transgenic mice deficient in ICAM-1 support the involvement of the adhesion molecules in the development of ischaemic damage. The homozygous knockout mice showed a reduction in infarct size and neurological deficit, had an increased survival and elevated blood flow in the infarcted hemisphere (Winquist & Kerr, 1997).

A potential cavaet of studies that investigate cell adhesion molecules and leukocytes particularly in focal cerebral ischaemia is that many of the studies employ the intraluminal monofilament model. This model has been criticised because the insertion and removal of

the occluding device can cause damage to the endothelium of the cerebral vessels which could lead to spurious results (Jean *et al.*, 1998). These criticisms have been somewhat answered by the fact that using another model of occlusion (autologous emboli in rabbits) where less damage may be caused to the endothelium (Bowes *et al.*, 1995). It should be appreciated that whilst observations regarding leukocyte infiltration may be an epiphenomenon of the model, other evidence regarding the inflammatory response (cytokine expression, microglial activation) do still indicate a potential role for inflammation in the pathophysiology of the disease.

GLIAL CELLS – MICROGLIA & ASTROCYTES

The term 'reactive gliosis' is used to describe the response of the endogenous glial cells to damage of neuronal tissues and is a topic of much discussion regarding its beneficial or detrimental role following brain injury (Kreutzberg, 1996; Giulian *et al.*, 1993). Glial cells respond to neuronal damage by under-going a number of characteristic changes and although may vary generally respond stereotypically and reproducibly although not necessarily at the same time (Streit *et al.*, 1999).

Microglia

Microglia often classed as the resident immune cells of the CNS and are the first non-neural cells to respond to CNS injury. They synthesise and secrete molecules such as cytokines and free radicals. These cells may be viewed as the co-ordinators of the inflammatory response in the brain as their activation is often followed by the infiltration of peripheral immune cells (Brint *et al.*, 1988; Kato & Walz, 2000; Kuge *et al.*, 1995). The role of microglia in the pathophysiology of cerebral ischaemia is discussed in detail in *Chapter 5*.

Astrocytes

Astrocytes are the predominant neuroglial cell type of the CNS and exist in two typical forms. Stellar-fibrillary astrocytes are normally located in the white mater whilst protoplasmatic astrocytes are located in the grey matter (Raivich *et al.*, 1999). As a cell type, they perform a variety of protective functions in the adult brain among which is protection against excitotoxic death by the removal of glutamate from the synaptic cleft. These cells buffer extracellular K^+ concentrations, process nutrients, regulate extracellular volume and release a host of neurotrophic factors (Nawashiro *et al.*, 2000). The astrocytes also provide physical support to the neighbouring neurones, meninges and vasculature and induce the formation of tight junctions on endothelial cells (Raivich *et al.*, 1999; Kato, *et al.*, 1994).

The astrocytes, like the microglial cells, respond to brain injury and their response depends on the extent of the trauma. Following injury the astrocytes rapidly synthesise glial fibrillary acidic protein (GFAP) and the protoplasmatic cells transform into fully stellarised, fibrillary astrocytes. This morphological change is controlled by cytokines expressed following injury (Raivich *et al.*, 1999). The characteristic hyperplasia and hypertrophy with increased GFAP staining that accompanies astrocyte activation has been reported in following cerebral ischaemic in the rat (Kindy *et al.*, 1992; Petito & Halaby, 1993) although others have reported a decline in GFAP reactivity (Chen *et al.*, 1993; Li *et al.*, 1995b). GFAP mRNA has been shown to be increased at 3 hrs post-occlusion, spread throughout the affected hemisphere within 3 days and have declined by 7 days (Yamashita *et al.*, 1996). Conversely, Liu *et al.* (1999) examined the astrocytic response following permanent MCA occlusion by measuring GFAP mRNA and protein expression and showed loss of GFAP mRNA as early as 3 hrs in the core of the infarct with an increase in expression in the peri-infarct area. Elevated expression remained for 25 days and was associated with gliotic scar formation

(Liu *et al.*, 1999). The astroglial activation has been associated with enhanced astroglial function and following insult these cells are thought to have an increased capacity for glutamate uptake, maintain water and ionic homeostasis and therefore may facilitate recovery following an ischaemic insult (Kato *et al.*, 1994). Another role suggested for the reactive fibrillary astrocytes is that they act as a physical barrier between healthy and damaged tissues (Raivich *et al.*, 1999). These suggestions are supported by the observation that GFAP null transgenic mice were more sensitive to permanent MCA occlusion with transient common carotid occlusion implying the astrocytes have a beneficial role in the ischaemic brain (Nawashiro *et al.*, 2000).

CYTOKINE EXPRESSION

Cytokines are expressed in the normal brain at levels that are hardly detectable. After injury however, various cytokines (interleukin-1β, interleukin-6 and tumour necrosis factor alpha) collectively called the pro-inflammatory cytokines, are known to be synthesised and released (Sharma & Kumar, 1998). The cytokines that are expressed in ischaemic tissue may play a significant role in the outcome of the compromised tissue. Cytokine expression in focal cerebral ischaemia is discussed in detail in *Chapter 6*.

iNOS

As discussed previously, NO has a notable role in the pathology of focal cerebral ischaemia. Of particular interest in a discussion centring on the inflammatory response to the reduction of blood flow to the brain is inducible or immunological nitric oxide synthase (iNOS). iNOS is not normally present in most cells but its expression is induced in pathological states, particularly those with associated inflammation (Nathan, 1997). Intracellular Ca^{2+} does not

regulate the enzyme unlike the other isoforms which are expressed in the post-ischaemic rodent brain, peaking at 12-48 hrs and is localised to the inflammatory cells and the cerebral blood vessels (del Zoppo *et al.*, 2000). iNOS has also bee observed in the brains of stroke victims supporting it role in the pathology of ischaemia (del Zoppo *et al.*, 2000).

MODELS OF CEREBRAL ISCHAEMIA

Stroke is a highly variable clinical condition and it is therefore difficult, despite advances in neuroimaging systems, to evaluate the dynamic biochemical events that follow ischaemia, determine the outcome of an insult or assess potential neuroprotective compounds in stroke patients. The cause, location, severity of an insult and the contribution of pre-existing systemic diseases are all uncontrolled variables in the patients that present with a stroke (Hsu, 1993). Therefore, studies to understand the pathology of the disease conducted in human patients would require large sample sizes to account for the variations (Macrae, 1992; McAuley, 1995). The use of animal models to study cerebral ischaemia has given enormous insight, into the pathological biochemical events that follow an ischaemic insult and also provides a means of investigating and assessing possible therapeutic neuroprotective strategies (Hunter *et al.*, 1995).

One advantage of using *in vivo* models to study cerebral ischaemia is the ability to perform controlled experiments and manipulate physiological variables. The use of standardised animal strains, alteration of the duration and severity of the insult and the ability to monitor and control physiological parameters such as blood pressure, gases, temperature and plasma glucose concentration is possible in a laboratory environment (Kirsch *et al.*, 1996). Animal models allow histopathological, biochemical and physiological investigation that require invasive surgery and direct access to the brain. They also allow assessment of events

occurring within minutes of an ischaemic insult (Ginsberg & Busto, 1989). Experiments to establish this type of data for humans would require patient compliance and would be difficult to support ethically. *In vitro* systems such as brain slices and tissue culture are limited methods of assessing the pathophysiology of stroke, as the disease is the consequence of abnormal perfusion of the brain. The recreation of abnormal perfusion requires the presence of intact vasculature as an integral component of an experimental system (Lipton, 1999). The use of *in vitro* systems of cerebral ischaemia offers an insight into aspects of the disease process, allows investigation in a simplified environment and provides a starting point for further investigation.

Stroke is a disease predominantly of middle or later life and is often complicated by other co-existing medical conditions. The experimental animals used to model stroke are generally young and may not truly represent the physiology of ischaemia in humans (Millikan, 1992; Recommendations for standards regarding preclinical neuroprotective and restorative drug development 1999). Consequently, some animal models have been developed to incorporate conditions such as hypertension that are associated with stroke (Ginsberg & Busto, 1989). Animals models of stroke model particular aspects of the disease and are essential for investiagtion of disease mechanisms and the evaluation of potential therapies and it should be noted that no stroke model exactly reproduces the human condition. The data from studies using different animal models has highlighted variations that exist between them (Oliff *et al.*, 1995; Duverger & MacKenzie, 1988). It is important to recognise that the involvement or contribution of one particular aspect of the pathophysiology could be an epiphenomenon of the stroke model. A standardised experimental approach and reproducible models are critical to understanding the cellular mechanisms involved in the pathology (Hsu, 1993). Variables such as rat strain, supplier, weight, age and anaesthetics used have an effect on the extent of ischaemic damage observed in different vessel occlusion methods (Brint *et al.*, 1988; Duverger & MacKenzie, 1988). Physiological variables such as blood pressure, brain and

body temperature, blood gases and plasma glucose concentration can influence neurological outcome following ischaemia and should, where possible, be monitored and kept with in normal physiological limits (Oliff *et al.*, 1995; Duverger & MacKenzie, 1988). Neuroprotective effects of compounds may also depend on the model of focal cerebral ischaemia and the species used for assessment (Recommendations for standards regarding preclinical neuroprotective and restorative drug development 1999; Takamatsu *et al.*, 1998).

Two other points should be considered regarding the use of animal models to model ischaemia. Like all biological organisms, species used experimentally are composed of rhythmical changing systems that may affect the response to the same stimulus depending on the time of day. In a study to determine the influence of circadian rhythms on infarct volume, Vinall *et al.* (2000) cautioned against the extrapolation of rat data to the human condition. They highlighted that the volume of infarct was correlated to body temperature linked to the animal's biological rhythms. Lesion volumes were greatest at the peak of activity at 04H00 and smallest (3 times smaller) at 16H00 (Vinall *et al.*, 2000). This observation of role of temperature in lesion formation is supported by studies with compounds like MK-801 that are known to reduce temperature and infarct size (Nakanishi *et al.*, 1994). Another consideration regarding the use of animal models for the assessment of ischaemic damage and the testing of potential neuroprotectants is that the majority of studies are performed in male animals because it negates that need to control for hormonal fluctuations associated with the female reproductive cycle (Roof & Hall, 2000). Carswell *et al.* (2000) demonstrated that female rats in pro-oestrus (high endogenous oestrogen) had smaller infarcts following MCA occlusion that animals in meta-oestrus (low endogenous oestrogen) (Carswell *et al.*, 2000). Vergouwen *et al.* (2000) however found that there were no significant gender differences but the discrepancies seen between these and other studies may be the result of the different occlusion methods and rat species used (Vergouwen *et al.*, 2000). Current epidemiological data suggest that stroke incidence and prevalence is shared equally between

the sexes however more women die of stroke than men (American Heart Association 2000). Analysis of clear-cut differences is difficult because of all the coincident variables in the clinical setting (Vergouwen *et al.*, 2000).

SPECIES USED FOR EXPERIMENTAL MODELLING ISCHAEMIA

Models of MCA occlusion were developed in primates as early as the 1930s and have been adapted and refined for use in other species (Peterson & Evans, 1937). A number of animal species ranging from small rodents to large sub-human primates have been used to investigate the disease (Ginsberg & Busto, 1989; Feuerstein & Wang, 2000). MCA occlusion experiments in large animals such as cats, dogs and primates have been performed for much of the century and have the advantage of possessing a gyrencephalic brain with extensive white matter like humans (Dewar *et al.*, 1999). Rodent models are now most widely used experimentally because of the low cost of the animals and associated reduction in cost of procedures that would increase with larger body size. The homogeneity of strains that results from inbreeding, similarities in the cranial circulation of rodents and man, small brain size suitable for fixation and subsequent histological studies make rodents ideal experimentally. There is also greater ethical acceptability in the West that makes rodents suitable subjects for research purposes (Ginsberg & Busto, 1989; Macrae, 1992). More commonly now genetically modified animals are used to study cerebral ischaemia. Transgenic mice have been used to investigate the effects of over-expression or deletion of a specific gene on infarct development following an ischaemic insult (Chabrier *et al.*, 1999; Chan, 1996).

The use of rodents routinely for the studying of MCA occlusions and gerbils for forebrain and global ischaemia does allow comparison of data from experiments performed by different laboratories but in so doing has decreased variability that is perhaps more representative of the clinical situation. Occlusion of the MCA (proximal to the

lenticulostriate vessels) results in an infarct in both the ipsilateral cortex and striatum and most closely resembles the anatomical distribution of the most common strokes seen in humans (Mattson, 1997). Different aspects of pathology may be highlighted using a wider range of species. It should however be constantly remembered that despite the modification and improvement of animal models they will never truly represent the clinical condition. They are however crucial to the understanding of the aspects disease pathology and not the disease itself.

GLOBAL VS. FOCAL ISCHAEMIA

The MCA and its penetrating arteries are prone to infarction in man (McAuley, 1995) and occlusion of this vessel is the most common underlying cause of ischaemic stroke in human (Nedergaard, 1988). Consequently, majority of animal models have been developed to mimic the clinical situation. Global ischaemia is a different disease and is modelled by brief near complete cessation of CBF followed by reperfusion with a very different resulting pathology (delayed cell death in the CA1 region of the hippocampus). The most popular global model is the 2 vessel occlusion in the gerbil although 2 and 4 vessel occlusions (sometimes incorporating hypotension) are also performed in the rat (Small & Buchan, 2000).

PERMANENT VS. TRANSIENT ISCHAEMIA

Models of cerebral ischaemia are also classed as permanent or transient. Clinically spontaneous recanalisation of the vessel has been reported in the first week post-insult in 24-50% of patients that results in the return of blood to the ischaemic zone (Saito *et al.*, 1987). However, more recent studies have shown that only between 4 and 17 % of patients displayed unassisted recanalisation (del Zoppo & Hallenbeck, 2000). Permanent occlusion in humans may result from increases in vascular resistance or plugging of the vessels by

components of the blood such as platelets (Marchal *et al.*, 1999). The permanent animal models have fundamental information about pathophysiological mechanisms responsible for tissue infarction, concepts of flow thresholds and assessing neuroprotectant drugs. Increased understanding of the clinical condition of stroke dictates that the consequences of reperfusion are investigated and therefore transient animal models have been developed and adapted to address this. The re-establishment of the blood to the hypo-perfused area is vital because it provides oxygen and substrates that the neurones require and it may salvage tissue that has not been irreversibly damaged. Alternatively reperfusion may have deleterious consequences and exacerbate the damage (Kristian & Siesjo, 1997) (*see Core, Penumbra & Reperfusion Injury*).

WHITE MATTER DAMAGE

The white matter of the brain is as susceptible to ischaemic damage as the grey matter. Focal ischaemia causes damage to both neurones and glial cells which is routinely evaluated by histology. The damage in the axons and the oligodendrocytes can also be assessed following an ischaemic insult by histochemical means but is often overlooked (Dewar *et al.*, 1999). Axonal ischaemic damage can be visualised by immunostaining for a variety of microtubule proteins of the cytoskeleton or by accumulation of molecules associated with axonal transport (Dewar & Dawson, 1995; Yam *et al.*, 1998). Damage to oligodendrocytes is marked by an upregulation of expression of the microtubule-associated protein, tau that has been used to show the sensitivity of these cells to permanent MCA occlusion *in vivo* (Stys, 1998).

EXPERIMENTAL MODELS OF FOCAL ISCHAEMIA

Each *in vivo* model of focal cerebral ischaemia has both strengths and weaknesses (***Table 1***). In order to achieve greater understanding of the pathology of ischaemia and develop new neuroprotective compounds, multiple models may give answers to a number of different questions by providing a more complete picture and an insight into the mechanisms of the disease. The guidelines for pre-clinical assessment of neuroprotectant in acute ischaemic stroke models suggests that different species (small and large animals) be included using models of both permanent and transient occlusion and that these studies be replicated by independent laboratories (Gorelick, 2000). Models of vessel occlusion in species where histological evidence and functional assessment (behaviour) can be assessed allows a more complete investigation of both the disease and potential therapies (Hunter *et al.*, 1995).

ELECTROCOAGULATION OF THE MCA

Robinson *et al.* (1975) were first to described a method ligating the distal portion of the rat MCA exposed by frontoparietal craniotomy to cause a focal ischaemic lesion (Robinson *et al.*, 1975). This model produced lesion volumes that were not reproducible and although occlusion affected the cortex, it often failed to affect the striatum. This method was modified by Tamura *et al.* (1981) using a sub-temporal craniotomy with removal of the zygoma to access the MCA proximal to the lenticulostriate branches of this artery and electrocoagulate it at that point (Tamura *et al.*, 1981a). The method was further modified by Shigano *et al.* (1985) leaving the zygoma intact reducing the eating difficulties experienced by the animals following destructive surgery (Shigeno *et al.*, 1985). Electrocoagulation has been shown to reduce cerebral blood flow to values below 25 ml .100 g^{-1} min^{-1} (Tamura *et al.*, 1981b), results in reproducible infarcts that affect both the cortex and striatum and has a low

mortality rate and was consequently historically widely used. Hemispheric infarct volumes of 100-150 mm^3 achieved by occlusion of the MCA proximal to the lenticulostriate branches in normotensive rats are representative of large infarcts observed in humans (McAuley, 1995).

Variation in the distribution pattern of infarct achieved using this method is attributed to differences in the site of the electrocoagulation (distal versus proximal occlusion of the MCA which varies in length) and the contribution of the lenticulostriate and other branches of the MCA. These factors can lead to differences in reproducibility of data obtained from this model making analysis of neuroprotection and other studies difficult. Alternative approaches have been attempted to improve the reproducibility of infarction including distal MCA occlusion combined with ligation of the ipsilateral common carotid (Brint *et al.*, 1988), concomitant MCA occlusion with bilateral common carotid artery occlusion (Chen *et al.*, 1986) and bilateral common carotid artery occlusion with hypotension (Smith *et al.*, 1984). Studies have been carried out using this model to assess the reproducibility of infarct size in different species and strains and incorporated risk factors such as hypertension (Duverger & MacKenzie, 1988). The drawbacks of the Tamura model include the severity of the surgery and the permanency of the occlusion that excludes investigation of the reperfusion. Modification of the method by using microclips, hooks or ligature snares allows reversal of the occlusion and examination of the events associated with reperfusion (Macrae, 1992).

A disadvantage of this model, which is associated with all involving major surgical procedures, is the use of anaesthetic agents. They may interfere with the evolution of the ischaemic lesion, aggravate or reduce the damage with prolonged administration and also complicate evaluation of neuroprotective compounds as they may be neuroprotective in their own right (Kirsch *et al.*, 1996). It is also possible that the properties of putative neuroprotectants may be altered by interaction with the anaesthetic compounds (Sharkey *et*

al., 1993; Sharkey & Butcher, 1995; Shapiro, 1985). Other disadvantages of this model include the exposure of the brain due to the craniotomy that may allow desiccation, thermal damage to the brain tissue, damage to the autonomic nerves of the vessel by cauterisation or clipping and possible instability of the thrombus induced by the electrocoagulation (Laing *et al.*, 1993; Longa *et al.*, 1989).

INTRALUMINAL OCCLUSION OF THE MCA

Koizumi *et al.* (1989) described a model of MCA occlusion in rats without craniotomy that can be either permanent or transient (Koizumi *et al.*, 1986). The method involves introduction of monofilament suture into the internal carotid artery (ICA) via the external carotid artery (ECA). The monofilament is then advanced intraluminally beyond the origin of the MCA where it occludes blood. Cerebral blood flow in the cortex and lateral striatum is reduced to between 4-19 ml.100 g^{-1}.min^{-1} (Macrae, 1992). This model does not require the demanding, potentially damaging surgery of the Tamura model and it has the advantage of allowing the restoration of blood flow by removal of the monofilament. Thermal damage and desiccation of the brain are avoided because craniotomy is not required and reperfusion can be instigated in conscious animals (Laing *et al.*, 1993; Longa *et al.*, 1989). A major disadvantage of this model is the unavoidable damage that occurs to the endothelial lining of the vessels. The lesion observed may therefore have a component resulting from vascular damage combined the consequences of the reduction in CBF. Additionally, damage to the vessel walls may be exacerbated on reperfusion having pathological consequences. The damaged vessels could also be a source of emboli that could occlude distal vessels (Macrae, 1992; Schmid-Elsaesser *et al.*, 1998).

There is considerable variation observed using the monofilament model (Macrae, 1992) and as with the Tamura model, studies have been performed to improve the reproducibility of

infarct volume. In the original Koizumi method, the common carotid artery was permanently ligated, the monofilament was coated with silicon at its distal end and advanced in to the Circle of Willis without occluding the anterior communicating artery (ACA). Longa *et al.* (1989) modified the monofilament by blunting the by heating and advancing it to the point where it reached the proximal segment of the ACA (Longa *et al.*, 1989). In addition, all the branches of the ECA and extracranial branches of the ICA were occluded to reduce bleeding. In 1993, Laing *et al.* conducted as study comparing the two methods above and concluded that the Koizumi method produced a more profound reduction in blood flow and more reproducible insult (Laing *et al.*, 1993). Nagasawa & Kogure (1989) described a method of transient MCA occlusion whereby a silicon rubber cylinder was attached to nylon surgical thread was used. Overall mortality was high in this study with 93 % of the animal dying within 48 hours (Nagasawa & Kogure, 1989). A further modification of the method was made by Belayev *et al.* (1996) who coated a monofilament with a flame-blunted end with poly-L-lysine (polycationic amino acid) (Belayev *et al.*, 1996b). They proposed that this encourage adhesion of the monofilament to the endothelium and appeared to improve the incidence, location and volume of infarct. The monofilament model and its variations is currently the most widely used experimentally (Schmid-Elsaesser *et al.*, 1998).

APPLICATION OF ENDOTHELIN-1 TO THE MCA

Attempts to minimise mechanical damage to the vessel by methods such as electrocoagulation and insertion of an intraluminal device has led to the development of models using a pharmacological approach to cause an ischaemic insult. Endothelin-1 (Et-1), a potent vasoconstrictor, applied to the MCA exposed by craniotomy was first described by Robinson *et al.* (1990) (Robinson *et al.*, 1990). The blood flow changes observed were comparable to the changes seen in the permanent electrocoagulation model. The volume of damaged tissue at 4 hrs following application of Et-1 was comparable to the same time point

in Tamura model. The advantages of this model are the lack of mechanical damage to the vessel and the ability to control the extent and duration of ischaemia by the altering the concentration of vasoconstrictor applied. There is still however the disadvantage of the need for craniotomy and its associated problems (Macrae, 1992). The use of the Et-1 may complicate the assessment of neuroprotective agents and it is important to establish interactions of such compounds with the vasoconstrictor and its receptors.

In alternative approach, Sharkey *et al.* (1993) applied Et-1 by stereotaxic perivascular microinjection to cause occlusion of the MCA (Sharkey *et al.*, 1993). The abluminal application of the Et-1 reduced blood flow in the ipsilateral hemisphere to values below 10 ml.100 g^{-1}.min^{-1}. The pattern of ischaemia was similar to that reported for the Tamura model of permanent MCA occlusion and produced lesions with volumes of damage comparable to large human infarcts (Sharkey *et al.*, 1993).

Advantages of this model are the ability to occlude any vessel for which the stereotaxic co-ordinates can be determined, the surgical procedures are less invasive than the sub-temporal approach and the application of Et-1 can be performed on conscious animals. In a further development of this model, Henshall *et al.* (1999) described the use of Endothelin-3 (Et-3) administered stereotaxically as a model of transient ischaemia (Henshall, 1999). The study compared the administration of Et-1 and Et-3 isopeptides and showed that the two isopeptides were equipotent in terms producing an ischaemic lesion but suggested that the Et-1 model was essentially a model of permanent ischaemia in which reperfusion is limited. The Et-3 model however provides a model in which controlled reperfusion is possible by administration of an Endothelin ET_A receptor antagonist FR139317. Reperfusion caused by the administration of the antagonist was less abrupt than with other models and may more closely mimic the reperfusion seen clinically. Disadvantages of the Et models

include the need for a craniotomy and needle tract damage associated with the stereotaxic injection.

THROMBOEMBOLIC MODELS OF MCA OCCLUSION

Photochemically induced occlusion

In this model of focal ischaemia, developed by Watson *et al.* (1985), occlusion of a specific vessel is achieved by producing an *in situ* thrombosis by photochemically stimulated platelet aggregation (Watson *et al.*, 1985). Despite early claims about the value of this model, based on an embolic occlusion and thus deemed to representative of the human condition, it is not widely used. The method requires the retraction of the scalp but not craniotomy, intravenous infusion of a photosensitive dye (Rose Bengal) with simultaneous irradiation of the desired area with a laser set at a specific wavelength (560 nm). The reaction of the intravascular dye and the light generates oxygen radicals which affect lipid molecules within the endothelium and blood elements giving rise to a microvascular platelet aggregation (Ginsberg & Busto, 1989).

An advantage of this model is that it avoids invasive surgery and there is a degree of control over damage and site of lesion by the manipulation of the wavelength and position of the light and the plasma concentration of the dye (Macrae, 1992). Disadvantages include rapid development of damage, significant breakdown of the blood brain barrier by the free radicals that are generated and vasogenic oedema. Although reperfusion can occur as the clot resolves itself there is little control over the event and as the embolism breaks up it may result in multiple infarction distally (Hunter *et al.*, 1995).

Autologous emboli

In the thromboembolic occlusion models, homologous or autologous blood clots (< 100 μm) are introduced into the internal carotid artery via retrograde cannula circulation placed in the common carotid arteries without disrupting the cerebral flow (Kudo *et al.*, 1982; Overgaard *et al.*, 1992; Zhang *et al.*, 1997). The emboli are produced by extravascular coagulation. This model has been used to produce strokes in larger species for many years and is attractive because of the little surgical manipulation that is required. The disadvantage is the lack of control over the distribution of the clots and consequently the uniformity and location of the infarcts (Hunter *et al.*, 1995). Adaptations of the model with clots of human blood have been used to test the efficacy of thrombolytic agents such as the human recombinant tissue plasminogen activator (Ginsberg & Busto, 1989). Studies using such models have illustrated the benefit of reperfusion and functional recovery when embolisms are removed by fibrinolytic therapies and highlighted the benefit of rapid thrombolysis and early recirculation (Yang *et al.*, 2000).

Microsphere embolisation

Calibrated microspheres (15-30 μm) are used as an alternative embolic material. Again little surgical manipulation is required but there is little control over the final destination of the spheres and consequently no uniformity of size and location of infarcts (Hunter *et al.*, 1995). Additionally an increase in cerebral oedema has been reported with this model (McAuley, 1995) and it is now seldom used.

In this thesis, the permanent and transient intralumnal monofilament and the Endothelin-1 models of MCA occlusion have been used. These models have been chosen as the monofilament model is widely used and appears to be most useful for the investigations into the inflammatory response. The Et-1 model is routinely used within the laboratory and provides a suitably different approach to vessel occlusion.

Table 1. Advantages & disadvantages of animal models of focal cerebral ischaemia.

METHOD OF OCCLUSION	ADVANTAGES	DISADVANTAGES	REFERENCE
Electrocoagulation/ ligation/clipping of the proximal MCA via sub-temporal craniotomy	▪ Reproducible infarct ▪ Volumes representative of large human lesions ▪ Reperfusion possible (except with electrocoagulation)	▪ Thermal damage and desiccation due to sub-temporal craniotomy ▪ Mechanical damage to vessel ▪ Possible post-surgical feeding problems ▪ Anaesthesia required	Tamura *et al.*, 1981a Shigano *et al.*, 1985
Intraluminal monofilament	▪ Relatively easy surgical procedure ▪ No need for craniotomy and associated problems ▪ No post surgery feeding difficulties ▪ Reperfusion can be achieved in conscious animals	▪ Damage to endothelium by insertion of monofilament ▪ Risk of puncturing the vessel ▪ Occlusion of ECA and its branches and pterygopalantine artery increasing infarct volume ▪ Anaesthesia required for insertion of monofilament although not necessarily for reperfusion	Koizumi *et al.*, 1985 Longa *et al.*, 1989
Abluminal application of Et-1 via sub-temporal craniotomy	▪ Controlled reperfusion possible dependent on dose of Et-1 applied ▪ No mechanical damage to vessel	▪ Sub-temporal craniotomy and associated problems ▪ Anaesthesia required ▪ Possible interaction of potential neuroprotectants with ET and receptors	Robinson *et al.*, 1990
Stereotaxic application of Endothelin isopeptides via dorsal craniotomy	▪ No mechanical damage to vessel ▪ Indwelling cannula can be used which allows administration of Et-1 to conscious animals ▪ Reversible model using Et-3 antagonist	▪ Craniotomy required although not as demanding as sub-temporal approach ▪ Needle tract damage ▪ Possible interaction of potential neuroprotectants with ET and receptors	Sharkey *et al.*, 1993 Henshall *et al.*, 1999
Photochemically induced thromboembolic	▪ No craniotomy required (depends on thickness of the skull) ▪ Site of occlusion chosen by irradiating desired target area	▪ Possibility of thermal damage by the light source ▪ Generation of free radicals which affect integrity of BBB ▪ Instability of clot and subsequent multiple infraction may occur ▪ Inability to control reperfusion	Watson *et al.*, 1985
Introduction of autologous/ heterologous emboli /microspheres	▪ Minimal surgical intervention ▪ Embolic model useful for assessing thrombolytic treatments	▪ Instability of clot and subsequent multiple infarction may occur ▪ Variation in infarct volume and location ▪ Inability to control reperfusion	Papadopoulos *et al.*, 1987 Zivin *et al.*, 1987 Kudo *et al.*, 1982

CURRENT TREATMENT & POTENTIAL THERAPIES FOR STROKE

The current prognosis for patients with stroke is worse than for myocardial infarction and many forms of cancer (Dyker & Lees, 1998). In the US, there is no approved medical treatment for acute stroke except tissue plasminogen activator (tPA/Alteplase, Genetech) which has limited use as it must be administered within 3 hrs of an ischaemic insult and carries a risk of intracranial haemorrhage (Dyker & Lees, 1998; del Zoppo *et al.*, 1997; Slow progress in the treatment of stroke 1998). In reality, only a few patients (5-8 %) qualify for treatment with in this short time window (Barone & Feuerstein, 1999)

An ideal neuroprotectant should reduce mortality in all types of stroke, irrespective of the location, severity and nature of the infarction and improve the functional and neurological prognosis of patients who survive (Giroux & Scatton, 1996). At all stages it must be remembered that volume of injured tissue following an ischaemia insult and the neurological outcome of the patient is dependent on the location of the occlusion, the duration of ischaemia, the collateral blood flow to the ischaemic zone and also the contribution of co-morbid illness (Black-Schaffer *et al.*, 1999). Many pharmaceutical companies have focused on the development of neuroprotective drugs for the treatment of stroke and promising results have been achieved in a vast number of pre-clinical studies with animal models and more than 100 clinical trials (***Table 2***). Thus far however, all Phase III clinical trials for many compounds have been discontinued because of adverse side effects or failed due to lack of efficacy (De Keyser *et al.*, 1999). The identification of a drug that could be administered to stroke patients requires a greater awareness of the condition and the facilities to provide rapid medical attention (Wester *et al.*, 1999). One of the difficulties with people who suffer a stroke is their failure to recognise that they are suffering a medical crisis, as the symptoms are not always obvious (Wang *et al.*, 2000; Heros, 1994). It is crucial for a rapid

accurate diagnosis to be made both in terms of administration of a therapeutic compound and also identification of suitable patients for inclusion in clinical trials for development of future compounds. Clinical evaluation of pharmacological therapies is a tricky issue irrespective of the disease at which the treatment is targeted and side effect and exposure to risk must be considered carefully (Albers *et al.*, 1998). To this end, the entire approach to stroke management has faced re-organisation with the issuing of guidelines and greater education (Kaste, 1997).

There are a vast number of therapies for acute stroke, both pharmacological and procedural, currently under development and many that have been abandoned. The clinical therapeutic goals of a neuroprotectant are three-fold: the rapid restoration of local cerebral blood flow, inhibition of the inflammatory sequealae of the ischaemic insult and the maintenance of neuronal integrity and function (del Zoppo *et al.*, 1997; Heiss *et al.*, 1999; Lutsep & Clark, 1999). The complex multi-faceted nature of the ischaemic cascade provides a number of points for therapeutic intervention and the key to successful neuroprotection may lie in combination therapies (Sacchetti *et al.*, 1997). Data has recently been published that supports this approach (Yang *et al.*, 2000; Heiss *et al.*, 1999; Asahi *et al.*, 2000). A therapeutic approach not included (***Table 2***) that has been evaluated and like many other compounds has had disparate results, focuses on oedema management following stroke (del Zoppo *et al.*, 1997). Osmotherapy using compounds such as mannitol showed only a temporary beneficial effect (Hossmann, 1982). Neuroprotective agents are aimed at preservation of neurones but improved functional outcome does depend on protection of cortical grey matter but also simultaneous protection of the associated white matter (Dewar *et al.*, 1999). Stroke therapies should thus be aimed at preservation of the neurones as complete functioning units. An alternative approach taken by pharmaceutical companies and clinicians to manage the disease is the use of preventative treatment that may help reduce the number of patients who present with a stroke (***Table 3***).

The Stroke Therapy Academic Industry Roundtable (STAIR) recommendations published in 1999 proposes guidelines for the evaluation of potential therapies. These include established drug dose, which is important as both pharmacokinetics and pharmacodynamics may vary considerably between species, the use of a variety of animal models and species as well as verification of potential compounds by different laboratories, the routine monitoring of physiological variables, meaningful assessment of outcome measures and awareness of sex differences in stroke (Recommendations for standards regarding preclinical neuroprotective and restorative drug development 1999). A number of recently published commentaries listed the trial related factors that have doomed human trials of neuroprotectants and re-enforced the need for and value of the STAIR recommendations (Gorelick, 2000). These included inappropriate time window, inappropriate patient selection, and lack of communication between preclinical researchers, industry and clinicians. Difficulties in assessing the severity of stroke and realistic outcome endpoints together with method of effective drug delivery were also highlighted as potential points that may cause a trial to fail (Obrenovitch *et al.*, 2000). If these issues are addressed and built into future clinical trial, perhaps an effective treatment for the disease will be found in the near future.

Table 2. Acute stroke therapy.

Mechanism of action	Subclass	Drug	Company	Status
Drugs to improve blood flow				
Anti-thrombotic		Heparin	-	Clinical use
		Nadroparin	Sanofi	III
		Tinzaparin	Leo	III
		Danaporoid	Organon	NCD
Antiplatelet		Asprin	-	?
		Abciximab	Eli Lilly & Co	II
Fibrinogen depleting		Ancrod	Knoll	III
Improve capillary flow		Pentoxifylline	Hoechst Marion Roussel	NCD
Thrombolytics		Streptokinase	Pharmacia	NCD
		Pro-urokinase	Abbott	III
		Tissue plasminogen Activator (Alteplase)	Genetech	FDA Approved
Neuroprotective Agents				
Calcium channel blockers		Nimodipine	Bayer	NCD
		Flunarizine		NCD
Free radical scavengers		Ebselen		III
		Tirilazad	Pharmacia & Upjohn	III
GABA antagonists		Clomethiazole	Astra USA	III
Glutamate antagonists	AMPA	GYKI 52466		
		NBQX		
		YM90K	Yamanouchi Inc.	NCD
		YM872	Yamanouchi Inc.	II
		ZK-200775 (MPQX)	Schering AG	NCD
	Kainate	SYM 2081		
	Competitive NMDA	CS 19755 (Selfotel)	Ciba-Geigy	NCD
	NMDA channel blockers	Aptiganel (Cerestat)	Boehringer-Ingelheim	NCD
		Dextrorphan	Roche	NCD
		Dextromethorphan		
		Magnesium		III
		MK-801 (Dizocilipine)	Merck	NCD
		NPS 1506	NPS	II
		SNX-111	Neurex	II

Glutamate antagonists	Glycine site antagonists	ACEA 1021	CoCeneSys	NCD
		GV150526	Glaxo Wellcome	III
	Polyamine site antagonists	Eliprodil	Synthelabo Lorex	NCD
		Ifenprodil		
Growth factors		Fibroblast Growth Factor (Trofermin)	Scios	III
Leukocyte adhesion inhibitor		Anti-ICAM antibody (Enlimomab)	Boehringer-Ingelheim Pharmaceuticals	NCD
		Hu23F2G	ICOS	II
Nitric oxide inhibitor		Lubeluzole	Janssen	NCD
Opiod antagonists		Naloxone		II
		Nalmefene	Baker Norton	III
Phosphatidylcholine precursor		Citicholine (CDP-choline)	Interneuron Pharmaceuticals	III
5-HT agonists		Bay x 3027	Bayer AG	III
Sodium channel blockers		Fosphenytoin	Parke-Davis	NCD
		Lubeluzole	Janssen/Glaxo	NCD
		619C89		?
Potassium channel opener		BMS-204352	Bristol-Myers Squibb	III
Unknown mechanism		Piracetam	UCB Pharma	NCD
		Lubeluzole	Janssen/Glaxo	

NCD – No clinical development in stroke

References The Internet Stroke Center 2000; Koroshetz & Moskowitz, 1996; Giroux & Scatton, 1996; del Zoppo *et al.*, 1997; De Keyser *et al.*, 1999; Slow progress in the treatment of stroke 1999.

Table 3. Procedures.

Procedure	Action/reason	Status
Carotid endarterectomy	Surgical therapy to reduce stroke risk	Major symptomatic and asymptomatic trials completed. Clear benefit of endarterectomy in symptomatic patients with >70% stenosis. Potential value of surgery for 50-69% (symptomatic) or 60-99% (asymptomatic) is much smaller and depends on surgical morbidity
Hyperbaric oxygen therapy	Treatment with oxygen under elevated atmospheric pressure	No randomised, multi-centre trials have been reported. Two small pilot studies of hyperbaric therapy do not support the use of this treatment in clinical stroke patients.
Hypothermia	Reduce the metabolic rate of the brain, which may extend time window for additional therapeutic intervention	No efficacy
Transfusion	Blood exchange for stroke prophylaxis in sickle cell anaemia	Trial was halted in September 1997 when interim analysis showed strong benefit for transfusion therapy in children with sickle cell anaemia.
Transluminal angioplasty	Angiographic procedure to dilate stenotic arteries.	Large multi-centre trials comparing angioplasty and endarterectomy are underway. CAVATAS trial (below) showed similar results with either treatment

References The Internet Stroke Center 2000; Koroshetz & Moskowitz, 1996; Giroux & Scatton, 1996; del Zoppo *et al.*, 1997; De Keyser *et al.*, 1999; Slow progress in the treatment of stroke 1999.

AIMS OF THESIS

The primary aim of this thesis was to investigate the contribution of inflammation in the pathophysiology of focal cerebral ischaemia by examining three animal models of MCA occlusion: rat permanent and transient monofilament models and the abluminal Endothelin-1 model. The initial hypothesis was based on observations that there are variations in animal models in terms of volume of damage and neuroprotective efficacy of compounds. It is speculated that these differences may be related to the inflammatory responses that occur post-insult.

CHAPTER 2

Materials & Methods

The methods described in this chapter are common to a number of subsequent chapters. Specific methods are detailed in individual chapters.

All experimental animals, unless otherwise stated, were male Sprague Dawley (SD) rats (275-315 g, aged 8-11 weeks) supplied by Charles River, UK. Rats were housed in a thermostatically controlled (22 ± 2°C), air-conditioned animal unit (12 hr day/night cycle; 50-60 % humidity) with food and water *ad libitum*.

All surgical procedures were performed under aseptic technique. Surgical instruments and non-sterile instruments were routinely immersed in cold sterilising fluid (Novasapa®, Willow Francis Veterinary). Surgical anaesthesia was induced by 5 % Halothane (Rhône Poulenc Chemicals Ltd) in a nitrous oxide: oxygen mix (70:30 v/v). Following induction of anaesthesia, animals were maintained at 1.5-2 % Halothane in the same gaseous mixture. Rectal temperature was monitored throughout all surgical procedures and animal body temperature maintained at 37.0 ± 0.5°C by a rectal probe attached to a thermostatically controlled heating blanket (CMA/Microdialysis AB150).

MIDDLE CEREBRAL ARTERY OCCLUSION

RAT PERMANENT MONOFILAMENT MODEL

Focal cerebral ischaemia was induced under halothane anaesthesia using the method described by Longa *et al.* (1989) with modifications (Longa *et al.*, 1989). The neck region of each animal was shaved in preparation for surgery using a Series 8900 cordless rechargeable animal trimmer (Wahl, USA). Midline incision and division of the omohyoid muscle exposed the left common carotid artery (CCA). The external carotid artery (ECA) was identified, dissected free from surrounding connective tissue and ligated distally with 6/0 silk suture (Ethicon, UK). A second 6/0 silk suture was tied loosely at the bifurcation of the ECA and the internal carotid artery (ICA). 4/0 silk suture (Ethicon) was placed around the ICA and tension applied to it to control bleeding. An angled atraumatic microvascular clip (6 mm micro-serrefine, Fine Scientific Tools) was placed across the CCA and a small incision made in the ECA at approximately the level of the origin of the occipital artery. The nylon monofilament (Ethicon), knotted at 18-19 mm, coated with poly-L-lysine (Sigma) and tip rounded near flame was introduced into the lumen of the ICA via the ECA. The suture was carefully advanced through the ICA into vasculature of the brain to a point where it occluded origin of the middle cerebral artery (MCA) (***Figure 6*** *&* ***Figure 7)***. Silk suture at the bifurcation of the ECA and ICA was tightened around the monofilament in order to secure it. The neck wound was sutured with 4/0 silk sutures (Ethicon) and the animal allowed to recover in an incubator where normothermia was maintained for 1 hr by which time it had fully recovered.

The post-operative care for each experiment was standard. The rats were placed in cages containing sawdust bedding only, returned to the animal unit and weighed daily. Animals

that were considered below the normal health status were supplemented with *i.p.* sodium chloride/glucose saline (0.18 % and 4 %, respectively) and/or a cereals soft based diet (e.g. Seven Cereals, Milupa UK). The supplementary care was only initiated 24 hrs post-lesion so as not to interfere with experimental procedures.

RAT TRANSIENT MONOFILAMENT MODEL

Surgery was performed as described for the permanent monofilament model (PMF). Two hrs post-occlusion unless otherwise stated, the animal was re-anaesthetised, the neck wound re-opened and the monofilament retracted to the point where the tip remained in the ECA and then cut. Blood flow in the ICA was visibly re-established in each case. Following surgery, the neck wound was sutured and the animal placed in an incubator to recover.

The effect of various occlusion times on the volume of damage in the transient model (TMF) was examined. The monofilament was withdrawn after 30, 60, 90 and 120 minutes and the brains processed as described previously. In a further experiment, animals subjected to a 30 min occlusion were allowed to survive for 14 days post-occlusion to investigate delayed cell death.

Evans Blue (2 % in saline, Sigma) was injected *i.v.* into anaesthetised animals 10 min prior to perfusion fixation. The dye was used to determine the placement of the monofilament in the vasculature of transient MCA occlusion animals. The dye stained the endothelium damaged by the insertion of the monofilament that was then visible on removal of the brain.

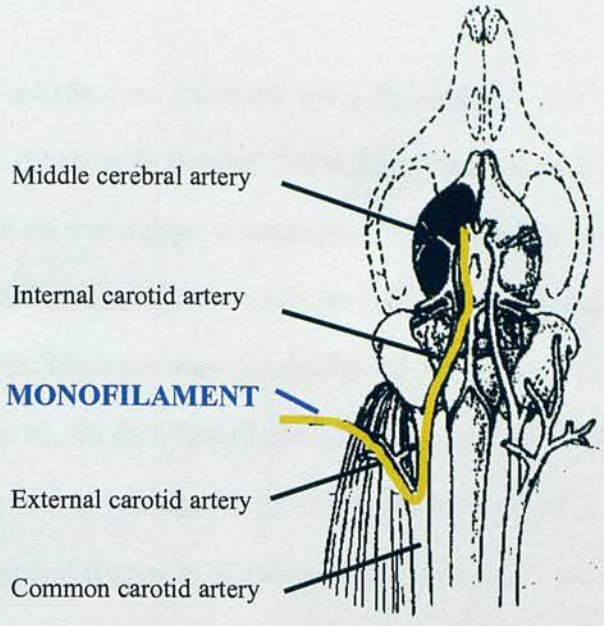


Figure 6. Placement of intraluminal monofilament for permanent and transient middle cerebral artery occlusion (Diagram modified from Longa *et al.*, 1989).

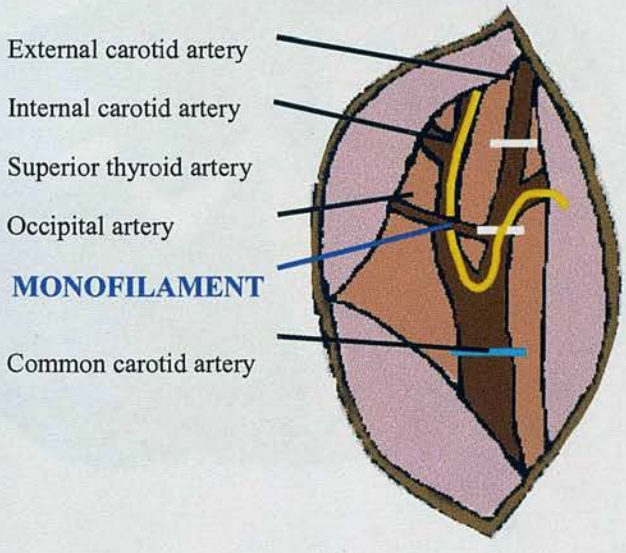


Figure 7. Surgical exposure of the arteries in the neck of the rat and insertion of the monofilament. White lines represent vessels closed by tied suture. Blue line represents position of atraumatic clip during MCA occlusion.

RAT ENDOTHELIN-1 MODEL

Occlusion of middle cerebral artery using Endothelin-1 was performed as described by Sharkey *et al.* (Sharkey & Butcher, 1995). Following induction of anaesthesia as described previously, the rat was placed in stereotaxic frame (Kopf Instruments) with tooth bar set at -3.7 mm. A midline incision was made on the scalp, the skin and periosteum retracted and Bregma located. The brain was exposed by a 2 mm craniotomy (0.9 mm anterior; 5.2 mm lateral to Bregma), the dura incised and a 26 gauge needle lowered slowly to pre-determined level (8.7 mm ventral to Bregma). Et-1 (150 pmols dissolved in saline) was injected in 3 µl over a 3 min period (***Figure 8***). Following the last injection, the needle was left *in situ* for 5 min and then slowly withdrawn. The scalp was sutured and the animal placed in an incubator to recover.

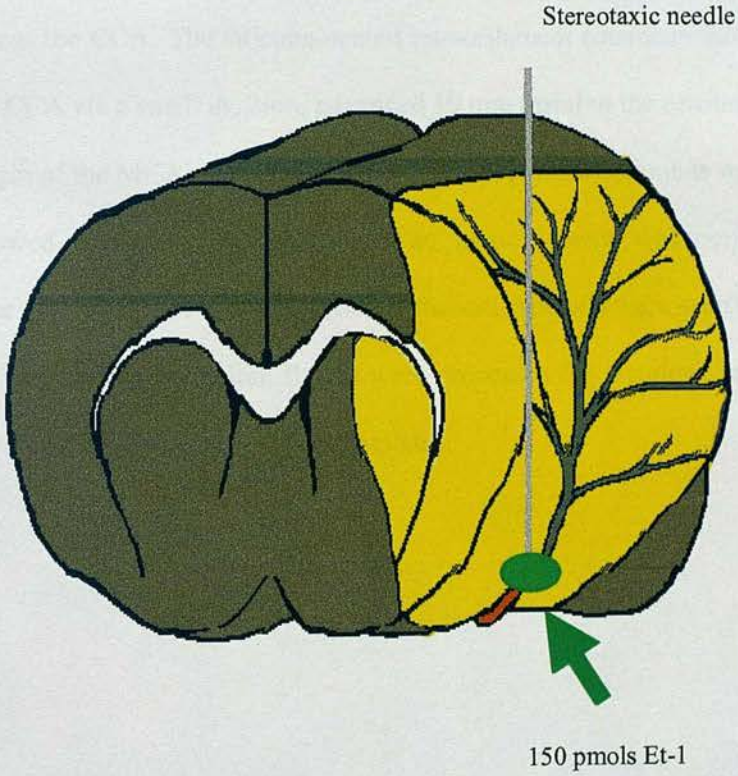


Figure 8. Occlusion of the MCA by stereotaxic application of Endothelin-1 (Sharkey *et al.*, 1993).

MOUSE TRANSIENT MONOFILAMENT MODEL

Dr Ailsa McGregor performed all mouse transient monofilament surgery. Male adult C57B/L6 mice (25-30 g, Charles River, UK) were anaesthetised as described in the rat monofilament surgery protocol. Focal cerebral ischaemia was induced by occlusion of the left MCA using an 8-0 nylon monofilament (Ethicon) coated with a mixture of silicone resin (Xantoprene, Heraeus Kulzer, Germany) and hardener (Elastomer Activator, Heraeus Kulzer, Germany) using the method previously described by Hata *et al.* (Hata *et al.*, 1998). Briefly, the left CCA, ECA and ICA and branches were exposed through a midline cervical incision. A 6-0 silk suture was loosely tied around the CCA proximal to the bifurcation of the ECA and ICA and a second suture tied around the ECA distal to the superior thyroid artery (STA). Both the STA and occipital artery (OA) were electrocoagulated and a microclip placed across the CCA. The silicone-coated monofilament (diameter 220 μm) was introduced into the CCA via a small incision, advanced 10 mm distal to the carotid bifurcation to occlude the origin of the MCA and secured (***Figure 9 & Figure 10***). Wounds were sutured and the animal allowed to recover in incubator. The monofilament was completely withdrawn under anaesthesia after 60 min to allow reperfusion. Animals were sacrificed 24 hrs post-occlusion by transcardiac perfusion. Brains were processed for histology and scored as described for the rat using mouse stereotaxic templates.

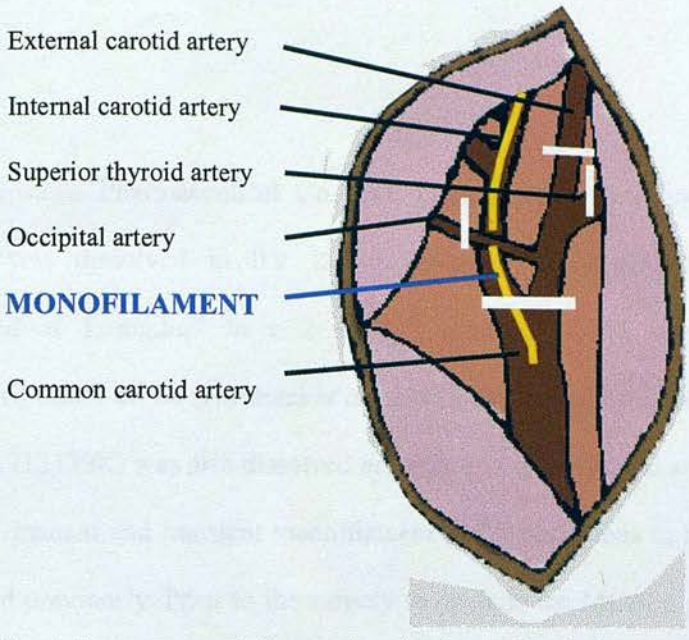


Figure 9. Surgical exposure of the arteries in the neck of the mouse and insertion of the monofilament. White lines indicated vessels closed by tied suture or diathermy.

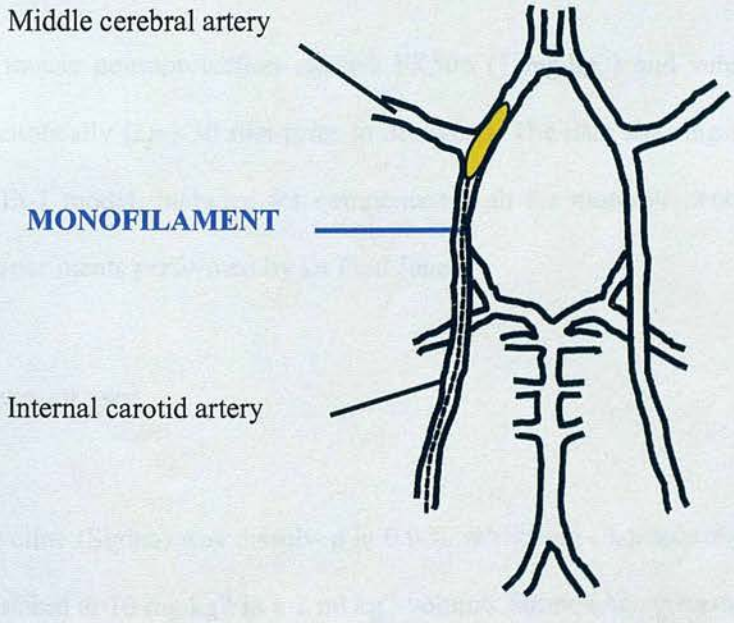


Figure 10. Placement of the monofilament in the vasculature of both the rat and mouse brain such that it occludes the origin of the middle cerebral artery.

NEUROPROTECTION STUDIES

FK506

FK506 (Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan; intravenous formulation Lot: 702144K) was dissolved in 0.9 % w/v saline (Aquapharm, Animalcare Ltd.) and administered at 1 $mg.kg^{-1}$ in a 2 $ml.kg^{-1}$ volume. FK506 vehicle (polyoxyethylene hydrogenated castor oil 60 (Hisatomi *et al.*, 1993), Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan; Lot: 713139K) was also dissolved in saline and administered at 1 $ml.kg^{-1}$ in a $2ml.kg^{-1}$ volume. Permanent and transient monofilament MCA occlusions in the rat were performed as described previously. Prior to the surgery to occlude the MCA, a polythene catheter was inserted into an exposed femoral vein. Drug and vehicle were infused intravenously (*i.v.*) 5 min post-occlusion. Following administration, the catheter was removed, femoral wound sutured and the animal allowed to recover as previously described.

In the mouse neuroprotection studies, FK506 (1 $mg.kg^{-1}$) and vehicle were administered intraperitoneally (*i.p.*) 30 min prior to occlusion. The data showing FK506 neuroprotection in the Et-1 model, included for comparison with the monofilament models, was obtained from experiments performed by Dr Paul Jones.

DOXYCYCLINE

Doxycycline (Sigma) was dissolved in 0.9 % w/v saline (Aquapharm, Animalcare Ltd.) and administered at 10 $mg.kg^{-1}$ in a 2 $ml.kg^{-1}$ volume. Saline (Aquapharm, Animalcare Ltd.) was administered as the control. Drug and saline were infused *i.p.* 2 hrs pre-occlusion. Permanent and transient monofilament MCA occlusions in the rat were performed as described

previously. A subsequent series of experiments were performed following the Clark *et al.* protocol (1997) (Clark *et al.*, 1997). Animals received either doxycycline (10 $mg.kg^{-1}$) *i.p.* or saline 30 min prior to occlusion followed by 10 $mg.kg^{-1}$ every 8 hrs until sacrificed 48 hrs post-occlusion.

ADMINISTRATION OF LIPOPOLYSACCHARIDE

Lipopolysaccharide (LPS; E. Coli 0111:B4; Sigma, Lot 78H4122) was dissolved in 0.9 % saline (Aquapharm, Animalcare Ltd.) and administered *i.p* at 2 $mg.kg^{-1}$. Animals were sacrificed by decapitation at 3 hrs post-administration, brains removed and cortex and striatum dissected out from both hemispheres. Samples were snap frozen and stored at -70°C until required.

HISTOLOGY

Unless otherwise stated, animals were sacrificed 72 hrs post-surgery by transcardiac perfusion. The animals were deeply anaesthetised by *i.p.* injection of sodium pentobarbitone (60 $mg.kg^{-1}$, Rhône Mèrieux). The thoracic cavity of the animal was opened and a needle connected to a peristaltic pump (Watson Marlow 502S) inserted through the apex of the left ventricle into the ascending aorta. A small incision was made in the right atrium. Animals were perfused initially with 20 ml heparinised (10 $IU.ml^{-1}$) phosphate buffered saline (PBS, *Appendix 1*) followed by 200 ml of 4 % paraformaldehyde (PFA) in PBS at a rate of 20 $ml.min^{-1}$ that would not cause any artifactual histological damage. Following perfusion, brains were removed, placed in 20 % sucrose in PFA/PBS solution and stored at 4°C for at least 24 hours. Brains were removed from sucrose/PFA/PBS solution, frozen at -42°C in isopentane and mounted in plastic embedding matrix (M1 embedding Matrix, Lipshaw) on

an orientating chuck. 20 μm cryostat sections (Bright OT/OFT Cryostat, Bright Instrument Company Ltd.) were taken at -20°C and placed on gelatin coated slides (5 %) and air dried at 20-30°C. Slides were stored at 37°C until stained.

Sections were placed in Histoclear (National Diagnostics) initially to remove lipid and rehydrated through an alcohol series before being placed in thionin (0.03 %, Sigma) for 20 min. Sections were differentiated in 0.2 % acetic acid, dehydrated through the alcohol series and mounted in xylene with DPX (distrene, plasticiser and xylene, BDH Chemicals Ltd, UK) (Drury & Wallington, 1967).

CALCULATION OF DAMAGE

Sections were examined under a light microscope (Leica, Galen) and the ischaemic damage annotated onto templates representing 9 pre-determined stereotaxic levels (*Appendix 8*). Templates were simplified from The Rat Brain in Stereotaxic Co-ordinates (Paxinos & Watson, 1986). The damage was quantified using a microcomputer imaging device (MCID M5+ Image Analyser, Imaging Research Inc.) and volume calculated according to the trapezoid model (Rosen & Harry, 1990). This method of damage calculation was validated before beginning experiments (*Appendix 5*).

TRAPEZOID RULE

$$V = \Sigma (x_{i+1} - x_i) [(y_i + y_{i+1})/2]$$

Where V is volume, x_i is the distance orthogonal to the plane of the *i*-th section and y_i is the cross sectional area of the *i*-th section (Rosen & Harry, 1990).

CALCULATION OF OEDEMA

The volume of oedema was also calculated from the original brain sections by computerised digital morphometry using the MCID computer. The ipsilateral and contralateral hemispheres at the 9 levels corresponding to those scored for damage were traced and the area of each determined (Overgaard & Meden, 2000). The volume of oedema was also calculated using the trapezoid rule. Differences in the volume of oedema (Δ) between the hemispheres expressed as a percentage.

CHAPTER 3

Comparison of stroke models

INTRODUCTION

The manipulation of lesion volume together with analysis of the differences that exist between and within models and species has provided an insight into and allowed investigation of the pathology of cerebral ischaemia. The evaluation of the volume of damage that occurs in a particular animal model is one of the most amenable endpoints of ischaemia experiments and as such is used routinely by many stroke research groups (Hunter *et al.*, 1995; Overgaard & Meden, 2000; Isayama *et al.*, 1991). To date, in studies assessing neuroprotective compounds, the only endpoint that has been routinely accepted is histological outcome although ideally neuroprotection should be manifest in improved functional outcome (Recommendations for standards regarding preclinical neuroprotective and restorative drug development 1999). The pathophysiological mechanisms involved in ischaemic tissue damage can be elucidated from animal models using pharmacological intervention or molecular biology approaches by observing the effect on the gross histological damage. Therefore, in studies that focus on the investigation of ischaemia and neuroprotection, the establishment of a consistently reproducible animal model of ischaemia coupled with a valid method of volumetric analysis is crucial. It is however also important to consider that in addition to histologically assessed neuroprotection, the functional outcome following an ischaemic insult can be a valuable indicator of cell survival and should not be overlooked (Hunter *et al.*, 1995; Feuerstein & Wang, 2000).

In accordance with the aim to investigate the inflammatory mechanisms associated with focal cerebral ischaemia, the intraluminal monofilament model, considered by some to be pro-inflammatory (Henshall *et al.*, 1999), was chosen as the primary animal model for investigation. This model, not previously established in Edinburgh, is widely used (Schmid-Elsaesser *et al.*, 1998), now highly reproducible (Belayev *et al.*, 1996b) and has a number of advantages over other models (*Chapter 1*). It is thought that the surgical procedure whereby the monofilament is advanced into and retracted (where necessary) from the cerebral vessels causes damage to the endothelium (McAuley, 1995). This fact is exploited by the use of Evans Blue to verify the correct placement of the monofilament (*Chapter 2*). As the endothelium is known to be involved in inflammatory processes demonstrated by cell adhesion molecule up-regulation, cytokine expression and platelet and leukocyte activation (del Zoppo, 1999), it is possible that any damage caused by the monofilament may contribute to the ischaemic damage observed and be responsible for some component of the ischaemic pathology. The hypothesis that inflammation is an important contributor to ischaemia but that there may be inflammatory differences between the different animal models of ischaemia and humans is supported by the differences in neuroprotective efficacy of anti-inflammatory strategies observed in different animal models (Chopp *et al.*, 1994; Garcia *et al.*, 1993; Zhang *et al.*, 1995). This hypothesis may also be supported by the failure of clinical trials of anti-inflammatory directed approaches such as the anti-ICAM-1 antibody of the Enlimomab Acute Stroke Trial (Lutsep & Clark, 1999).

Re-establishment of blood flow to ischaemic tissue may be a key component of the inflammatory response to cerebral ischaemia. Reperfusion may exacerbate the damage if the endothelium of the occluded vessels have been damaged, both as a consequence of the biochemical events of the induced ischaemia (Stanimirovic & Satoh, 2000) and possibly by the surgical manipulation. Del Zoppo *et al.* (2000) proposed that the cerebral microvasculature is not only the site of initiation of an inflammatory response during

ischaemia but suggested that there are events that occur there are not recovered on the return of blood to the ischaemic tissue (del Zoppo *et al.*, 2000). An inflammatory response could therefore make a definite contribution to the ischaemic damage and be distinct from that attributable to reperfusion. This suggestion does however not address the direct damage cause by the surgical manipulation. This provided the rationale for examining the differences between permanent and transient occlusions in the same model and the additional comparison with the Et-1 model, which does not directly damage the endothelium and may be less inflammatory. These studies could address the question of the validity of using a particular model if the surgical procedure employed introduces an aspect of pathology that may be an epiphenomenon associated with the model used. Such studies could also support the case for evaluation of ischaemia and neuroprotectants efficacy in multiple stroke models (Takamatsu *et al.*, 1998). Additionally, the dissection of the spatial and temporal relationships of the inflammatory response can provide perhaps novel approaches for therapeutic intervention and salvage of brain tissue.

The initial experiments conducted were aimed at establishing a reliable protocol for the intraluminal monofilament model. These studies were also used to develop and validate new stereotaxic templates and the computerised digital morphometry for lesion volume analysis compared with the 'cut and weigh' method used previously. Once the permanent monofilament model was established, the model was compared with the transient model and also the Endothelin-1 model established by Sharkey *et al.* (1993) and routinely used for stroke studies within the laboratory (Sharkey *et al.*, 1993). The neuroprotective efficacy of FK506 was assessed in both models. FK506 was chosen both because its neuroprotective efficacy was demonstrated originally in the Endothelin-1 model within this laboratory but additionally as an immunosuppressant compound it may potentially affect components of an inflammatory response. If differences in the models were due to differences in the

inflammatory response to the insult could be reflected in the extent of neuroprotection achieved.

In addition to the investigation of the volume of tissue damage it was decided to investigate the volume of oedema in all three models. The swelling of the brain complicates the disorders of the CNS and is therefore of clinical importance, is associated with inflammation and could be a potential therapeutic target (*Chapter 1*). The measurement of the extent to which the volume of the brain increases following an insult reflects the severity and the extent of damage (Plum, 1983). It is however unclear if oedema formation following an ischaemic insult is a cause of tissue damage that leads to infarction or the consequence of tissue dysfunction precipitated by the reduction in blood flow i.e. cause or consequence of an ischaemic insult.

MATERIALS & METHODS

The materials and methods for the data presented in this chapter are described comprehensively in *Chapter 2*. Volume of damage and oedema are expressed as the mean ± standard error of the mean (sem) in mm^3 and as difference (Δ) between ipsilateral and contralateral hemispheres as a percentage, respectively. Statistical analysis was performed using SigmaStat. For each experiment analysis of variance (ANOVA) with *post hoc* Student-Newman-Keuls test was performed.

RESULTS

VOLUME OF DAMAGE

Ischaemic brain damage was readily discernible in the thionin stained sections 72 hrs post-occlusion by light microscopy (***Figure 11***). In all models, the lesion affected lateral parts of the frontal cortex extending through the parietal and into insular cortex rostrally and through temporal and into occipital cortex caudally. There was evidence of infarction within the dorsolateral caudate nucleus (striatum) (***Figure 12***). This pattern of damage is typical of an MCA occlusion and encompasses the entire vascular territory of the vessel. This is reflected in the volume of damage seen in the three models. In the PMF model, the overall volume of damage was 239 ± 20 mm^3. The overall volume of damage in the TMF was 249 ± 10 mm^3 and that in the Et-1 model was 213 ± 26 mm^3. Statistical analysis of the data from the three models revealed that there was not a significant difference in the volume of damage between the three. There was no evidence of damage in PMF and TMF monofilament sham occlusion groups. A small amount of damage (16 ± 6 mm^3) in the Et-1 sham group was associated with the needle tract of the stereotaxic injection and was significantly different from the Et-1 induced occlusion ($p < 0.05$) (***Figure 13***).

EVOLUTION OF INFARCT WITH INCREASING OCCLUSION TIMES

The concept of the ischaemic penumbra and the 30 min insult data presented by Du *et al.* (Du *et al.*, 1996) suggested that an ischaemic lesion should develop with time. Reperfusion injury would also suggest that transient models should have larger lesions. Investigation of the volume of damage produced by different periods of MCA occlusion by intraluminal monofilament revealed that 30 and 90 min of transient ischaemia caused no

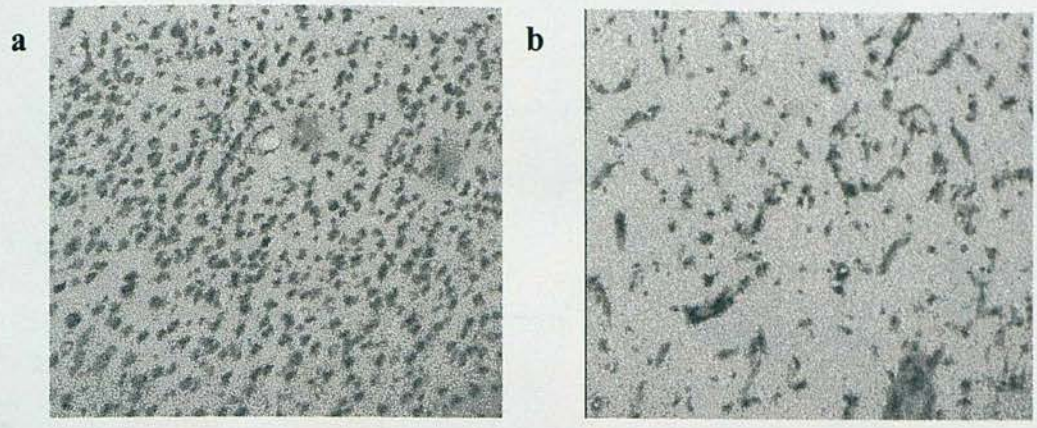


Figure 11. Thionin stained cryostat section (20 μm) showing the non-ischaemic contralateral hemisphere (a) and ischaemic damage in ischaemic ipsilateral hemisphere (b) following permanent monofilament occlusion of the middle cerebral artery.

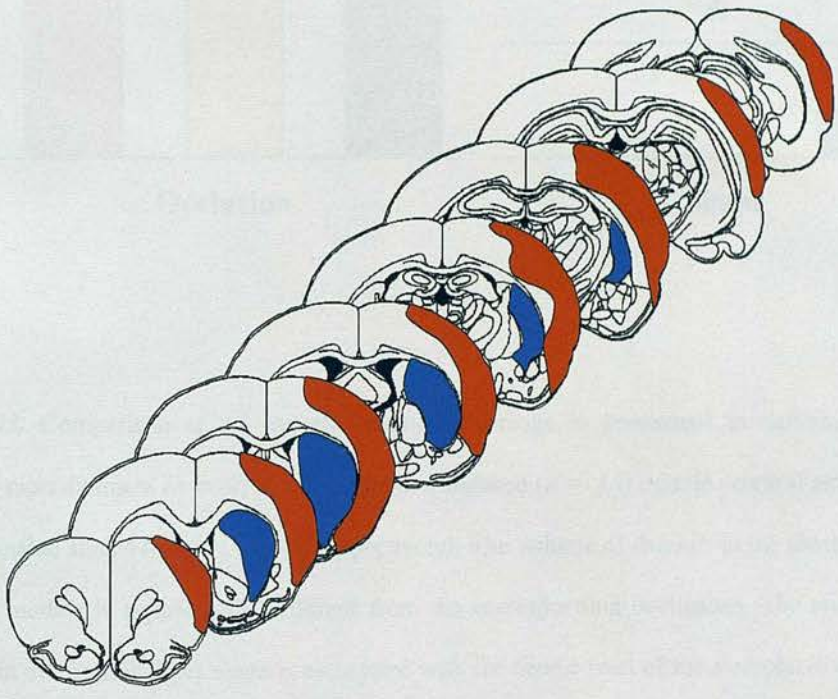


Figure 12. Schematic representation of the classical distribution of infarction of the cortex (red) and striatum (blue) following middle cerebral artery occlusion in the rat (Sharkey & Butcher, 1994).

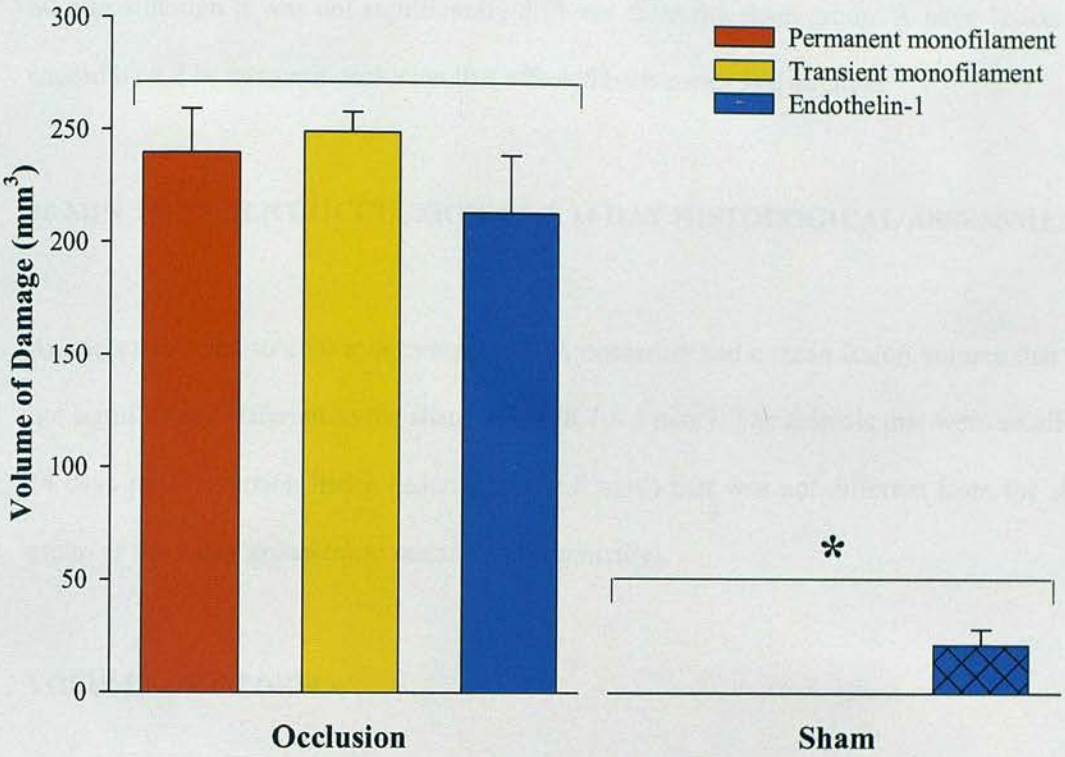


Figure 13. Comparison of the overall volume of damage in permanent monofilament (n = 9), transient monofilament (n = 8) and Endothelin-1 induced (n = 14) middle cerebral artery occlusions and associated shams (n = 4, 5 and 7 respectively). The volume of damage in the sham occlusions of all three models is significantly different from the corresponding occlusions. The small volume of damage in the Endothelin-1 sham is associated with the needle tract of the stereotaxic injection. Data represent mean ± sem. (* $p < 0.05$, ANOVA with *post hoc* Student-Newman-Keuls test).

significant damage to the brain parenchyma ($p < 0.05$ %) (***Figure 14***). However, 120 min of transient occlusion produced a large lesion whose overall volume is indistinguishable from that of a permanent occlusion (***Figure 13***). Damage was initially observed in the striatum as early as 30 min post-occlusion. Cortical damage was observed at 60 min although it was not significantly different from the sham group. A large lesion was caused by a 2 hr transient occlusion that affected both cortex and striatum.

30 MIN TRANSIENT OCCLUSION – 3 & 14 DAY HISTOLOGICAL ASSESSMENT

Animals subjected to a 30 min transient MCA occlusion had a mean lesion volume that was not significantly different to the sham group (8.7 ± 6 mm^3). The animals that were sacrificed 14 days post-occlusion had a lesion (2.5 ± 2.8 mm^3) that was not different from the sham group of the 3 day group (*data not shown graphically*).

VOLUME OF OEDEMA

Temporary occlusions are often associated with larger lesions and a greater degree of oedema (McAuley, 1995). The data from these experiments show that there was not a statistically significant difference in the volume of oedema in all three models (***Figure 15***). The volume of oedema in the Et-1 model tended to be smaller but not significantly different. There was a significant difference ($p < 0.05$) between MCA occlusion and sham-operated animals. Again, the small volume of oedema in the Et-1 sham operated group is associated with damage cause by stereotaxic application of the vasoconstrictor.

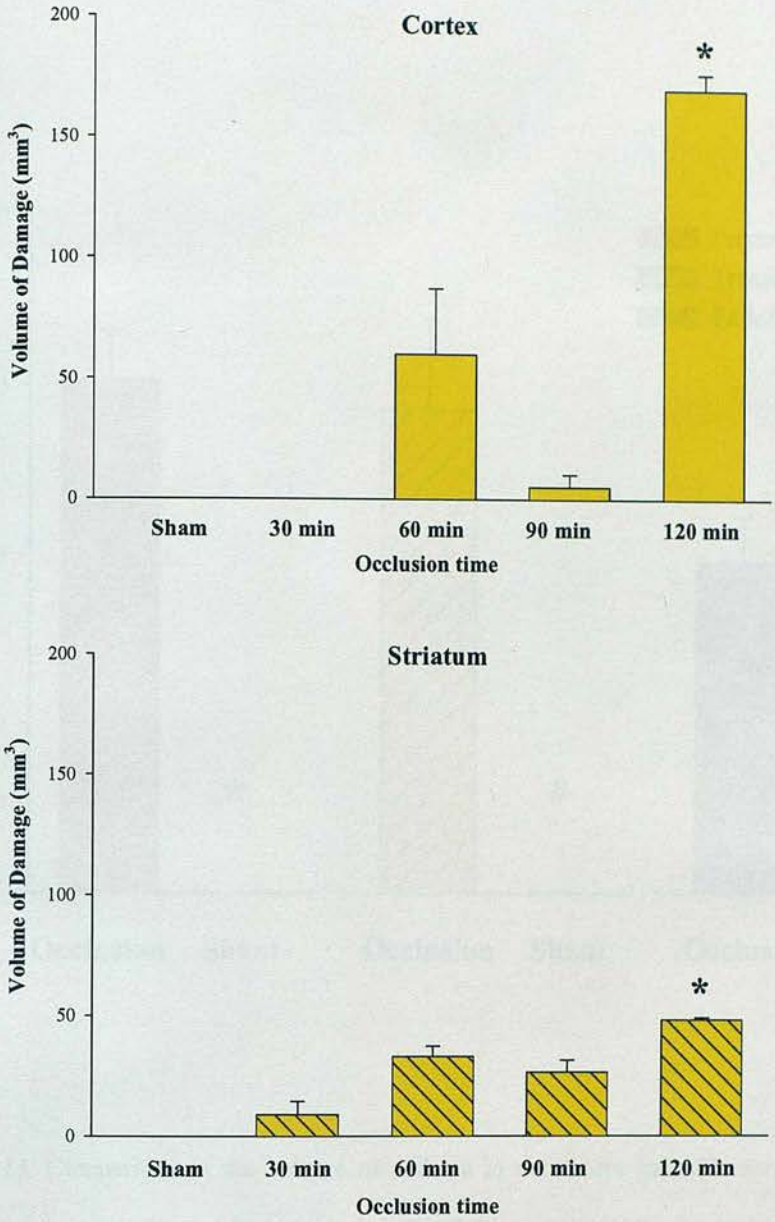


Figure 14. Comparison of the volume of damage in the cortex and striatum in the transient monofilament model associated with increasing occlusion times (n = 5, 9, 12, 12 and 8 respectively). Only a transient occlusion of 120 min produces damage in the cortex and the striatum that is significantly different from the sham group. Data represent mean ± sem. (* $p < 0.05$, ANOVA with post hoc Student-Newman-Keuls test).

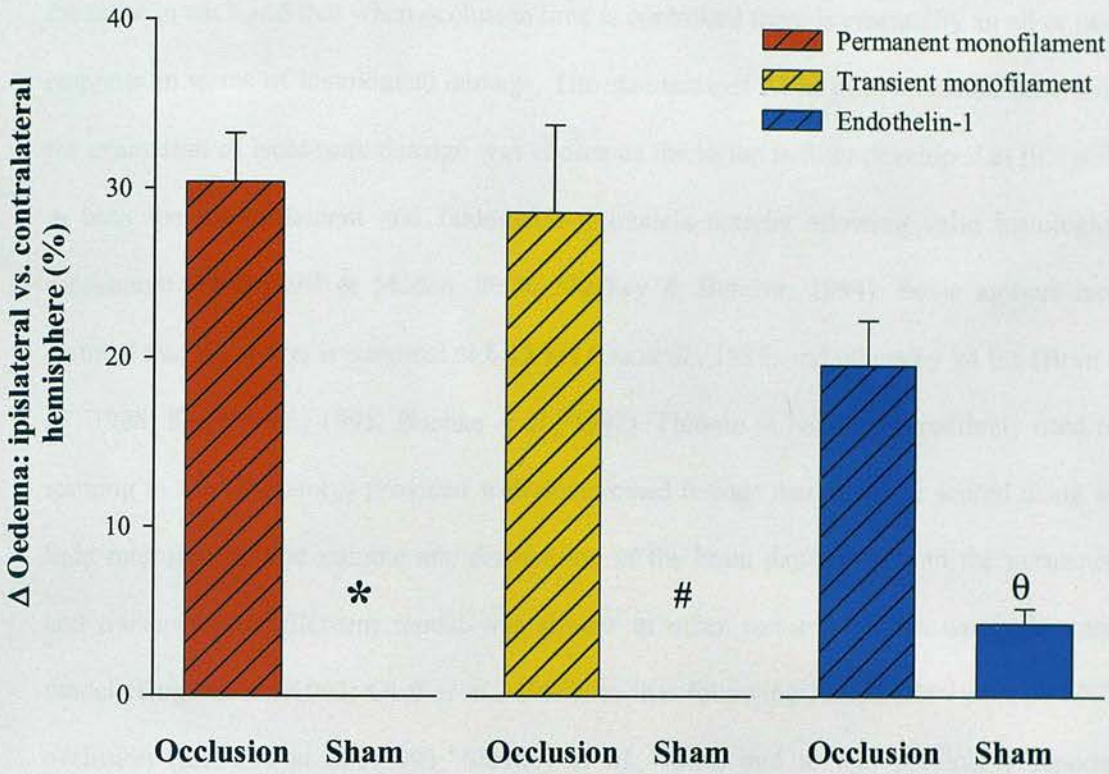


Figure 15. Comparison of the volume of oedema in permanent monofilament (n = 9), transient monofilament (n = 8) and Endothelin-1 induced (n = 14) middle cerebral artery occlusions and associated shams (n = 4, 5 and 7 respectively). Oedema is expressed as the percentage difference in the volume of the ipsilateral hemisphere compared the contralateral hemisphere (Δ). The small volume of oedema in the Endothelin-1 sham is again associated with the needle tract damage. Data represent mean ± sem. (*, #, θ $p < 0.05$, ANOVA with post hoc Student-Newman-Keuls test).

DISCUSSION

In addressing the role of reperfusion injury in cerebral ischaemia, three models of MCA occlusion were compared. The data shows that the size and distribution in all three models is the same in each and that when occlusion time is controlled there is essentially an all or none response in terms of histological damage. The standardised 72 hr post-occlusion time point for evaluation of ischaemic damage was chosen as the lesion is fully developed at this point in both the monofilament and Endothelin-1, models thereby allowing valid histological assessment (Overgaard & Meden, 2000; Sharkey & Butcher, 1994). Some authors have claimed that the lesion is maximal at 6-12 hrs (Du *et al.*, 1996) and others by 24 hrs (Brint *et al.*, 1988; Kuge *et al.*, 1995; Buchan *et al.*, 1992). Thionin (a Nissl stain routinely used for staining in the laboratory) provided well-demarcated lesions that could be scored using the light microscope. The volume and distribution of the brain damage in both the permanent and transient monofilament model was similar to other research groups using the same model (Kuge *et al.*, 1995; Oliff *et al.*, 1995), to that following Endothelin-1induced MCA occlusion (Sharkey *et al.*, 1993; Sharkey *et al.*, 1994) and to that previously reported following surgical occlusion (Tamura *et al.*, 1981a). Initial studies established the intraluminal monofilament model as a reproducible and valid model for investigating the pathophysiology of focal cerebral ischaemia. The length of suture chosen (18-19 mm) and method of preparation (poly-L-lysine coated with monofilament tip rounded in a flame) produced the classical pattern of damage seen following surgical occlusion of the MCA and to that seen in the Endothelin-1 model (Tamura *et al.*, 1981a; Sharkey & Butcher, 1994). A small study was performed comparing poly-L-lysine coated and uncoated Ethilon sutures and proved there was no difference in terms of lesion volume but the lesions in experiments with uncoated sutures were more variable (*data not shown*). Poly-L-lysine is polycationic polymerised amino acid that adsorbs to surface thereby increasing adhesive forces between

the monofilament and the vascular endothelium (Belayev *et al.*, 1999). These observations were comparable to those of Belayev *et al.* (1996) who performed a similar set of experiments. Coated monofilaments produced more consistently reproducible lesions although the lesions volumes were slightly greater (Belayev *et al.*, 1996b). The mean lesion volume in the monofilament model was similar in comparison to data from experiments using different models (Takamatsu *et al.*, 1998; Kuge *et al.*, 1995; Herz *et al.*, 1998). A further refinement of the transient monofilament model was the use of Evans Blue to confirm the correct placement of the monofilament in the vasculature.

The surgical protocol used to occlude the MCA was performed such that when recirculation was instigated in the transient model, blood flow through the ipsilateral CCA was re-established. Some protocols for performing the monofilament surgery, including the original described by Koizumi *et al.* (1986), rely on collateral blow flow from the Circle of Willis for reperfusion as the CCA is permanently ligated (Koizumi *et al.*, 1986). In these models, it is questionable whether pre-ischaemic blood flow levels are achieved in the ipsilateral hemisphere by collateral blood flow although data from the mouse monofilament used in house suggests that there is adequate reperfusion (*Dr A. McGregor personal communication*). In the current experiments, return of blood flow through the ipsilateral CCA was confirmed visibly in each experimental animal. Although successful occlusion of the MCA was ultimately confirmed by histological analysis, neurological deficits (rotation, limb weakness etc) following surgery were also noted for each animal. In the permanent model, the correct placement of the monofilament in the Circle of Willis such that it occluded the origin of the MCA was confirmed when the brain was removed from the skull. In the transient model, Evans Blue injected into the animal prior to perfusion fixation established correct placement of the monofilament in the cerebral vasculature. The damaged endothelium corresponding to the position of the suture retained a blue stain after fixation and was clearly visible when the brain was removed. The data obtained from these

experiments was also used for power analysis to estimate the group size of 5, 4 and 14 for the PMF, TMF and Et-1 models respectively that would be required to detect an 50 % reduction in ischaemic volume with 80 % power.

The comparison of the three different animal models revealed no difference in either the volume of damage or oedema. This observation is in agreement with an investigation by Zhang *et al.* (1994). The temporal profile of ischaemic tissue damage in both the permanent and transient (2 hr) monofilament model was examined in the Zhang *et al.* (1994) studies and revealed that the lesion in the transient occlusion group was initially smaller than that of the permanent group but by 48 hrs the size and features of the final lesion were indistinguishable. The temporal profile of the cellular response in the two models was not identical and it appeared that there was slower evolution of damage in the cortex of the transient model during early reperfusion (Zhang *et al.*, 1994). This observation could support the view that reperfusion does have a beneficial role but it is balanced by factors such as duration and severity of ischaemia (Duverger & MacKenzie, 1988).

The study showing spatial development of tissue damage caused by increased occlusion time confirmed observations reported by others (Memezawa *et al.*, 1992). Short periods of ischaemia (30 min) in the monofilament model followed by reperfusion have previously been shown by others to produce infarcted areas in the core of the lesion (striatum) and 60 min occlusions to produce cortical damage (Du *et al.*, 1996). These data are consistent with current observations in this thesis in terms of size and distribution of the lesion and suggest that early reperfusion prevents rather than promotes extension of the infarct. The small volume of damage seen in the cortex of the 60 min occlusion animals in the experiments in this thesis is not significantly different from the sham data and highlights biological variation of experimental subjects. In the Memezawa study, it was also demonstrated that reperfusion

after 90 min occlusion was beneficial however after 2 hrs return of blood to the ischaemic zone failed to salvage more tissue (Memezawa *et al.*, 1992).

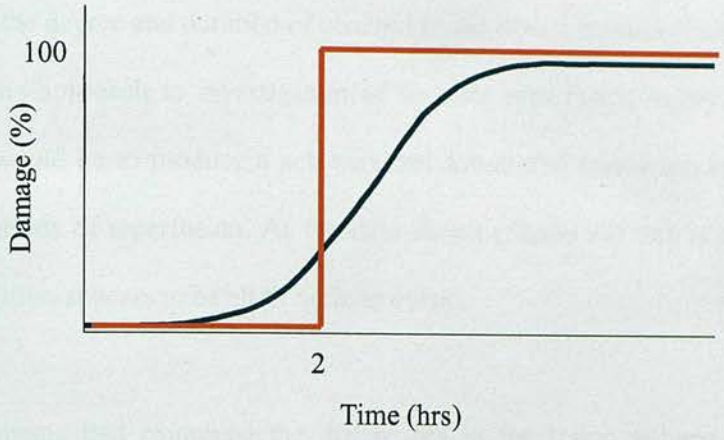


Figure 16. Theoretical maturation of an ischaemic lesion (black line) and experimental data (red line).

Reperfusion injury occurs in other organs such as the heart (Park & Lucchesi, 1999; Grech *et al.*, 1995; Lucchesi, 1990) and other peripheral organs such as the liver (Tredger, 1998; Blennerhassett *et al.*, 1998). The data presented in this thesis suggest it does not occur within the brain following ischaemia and thus does not increase lesion size. It is possible that the lesion caused by 2 hrs of vessel occlusion is maximal and therefore no more damage can occur in the compromised territory. As with all comparisons with the data produced by other research groups, care should be exercised. Experimental protocols and details such as blood pressure and anaesthetic used can influence the volume of lesions produced by an insult and may account for differences observed. For example, in the Memezawa *et al.* study (1992), Wistar rats and a different monofilament occluder used (Memezawa *et al.*, 1992). Careful control of surgery and animal physiology during anaesthesia should result in a lesion that is confined to the vascular territory of the MCA. Whilst reperfusion has been shown to have benefits in reducing lesion size (Young *et al.*, 1997), others have reported an increase in the

volume of damage following reperfusion (Aronowski *et al.*, 1997; Dietrich, 1994; Takamatsu *et al.*, 2000). The efficacy of reperfusion depends on the rapidity of the restoration of blood flow because the survival of ischaemic tissue decreases exponentially with time but is also a function of the degree and duration of cerebral blood flow reduction (Jiang *et al.*, 1998). The most obvious approach to investigation of the role reperfusion injury following cerebral ischaemia would be to produce a sub maximal lesion and assess lesion volume following different periods of reperfusion. As the data shows (***Figure 14***) this is not possible, as the lesion formation appears to be all or nothing event.

The experiments that examined the differences in the lesion volume at 3 and 14 days post-occlusion contrasted data from a previously published study. Du *et al.* (1996) showed the development of a small lesion assessed 3 days after a 30 min MCA occlusion with bilateral CCA occlusion. However, when assessed 14 days post insult the lesion had developed and was indistinguishable from a lesion caused by 90 min occlusion of the vessel. Since the development of the lesion was delayed by 14 days it was suggested to be the result of apoptosis and this model could be used to study cell death in a purely apoptotic environment (Du *et al.*, 1996). This observation could not replicated in either the Endothelin-1 or Endothelin-3 (Henshall, 1997) or in the mouse model (*Dr A. McGregor unpublished data*). It is possible that the combination of MCA occlusion with bilateral CCA is a more severe insult than MCA occlusion only. Although not causing necrosis the disruption in blood flow caused by this method does stimulate the apoptotic machinery in the cell.

The examination of oedema in the three different models in this thesis was done by computerised digital morphometry to compare the volume of ipsilateral to contralateral hemispheres in similar manner to that described recently by Overgaard *et al.* (2000). The method of assessing the oedema associated with the ischaemic damage has a number of

caveats such as different fixation methods on the volume (Overgaard & Meden, 2000; Brint *et al.*, 1988) but does however provide an indication of the consequences of an ischaemic insult. The principle conclusion drawn from the data confirms that oedema occurs following an ischaemic insult and that the extent of oedema is related to the volume of damage i.e. the greater the damage the greater the oedema (Grome *et al.*, 1988). Analysis of the difference in volume of the between the hemispheres should be treated with some caution because as with all increases in volume within an enclosed space, in this instance the skull, there may be some distortion of the contralateral hemisphere which is used as a control. Cao *et al.* (1994) used a method of calculation of oedema similar to the one used in this thesis. Lesion volume was established by TTC staining and expressed as a percentage of the ipsilateral hemisphere to eliminate the contribution of oedema. The volume of both the contralateral and ipsilateral was calculated using computerised image analysis of the sections and the difference, deemed to represent oedema, was expressed as a percentage (Cao & Phillis, 1994). Other methods used to calculate oedema volume include the dehydration method where the pre- and post-dehydration weights of the brain hemispheres and gravimetry, where the specific gravity of the hemispheres is calculated and related to water content (Menzies *et al.*, 1993; Schwab *et al.*, 1997). Differences in oedema in the monofilament model was examined by Koizumi *et al.* (1989) who demonstrated that oedema was noticeable earlier in the model with a reperfusion element (Koizumi *et al.*, 1986). The differences seen in the volume of oedema in the monofilament models and the Endothelin-1 model, although not significant, may be a consequence of the method of occlusion. It may be speculated that application of Endothelin-1 peptide to a cerebral vessel may have pharmacological effects on water transport mechanisms (Hughes *et al.*, 1992; Phillips & Yeates, 2000) although the area of such an effect is probably negligible in relation to the infarct volume. Alternatively, the profound vasoconstriction produced by the Endothelin-1 may limit water transport.

The appraisal of the method of volumetric analysis previously used in the laboratory and the new computerised method based on quantitative morphometry proved that the new method was valid (*Appendix 5*). There was no significant difference in the volume of damage calculated between the previously used 'cut & weigh' method and the computerised method. The computer-based method was however more efficient and data could easily stored and accessed. The use of the templates negated the need to correct the infarct volume to account for oedema required if morphometric volume analysis was performed using the histological sections. The trapezoidal rule was chosen to calculate the volume as it accounted for the unequal distances between the template levels chosen (Rosen & Harry, 1990). There are a variety of methods that are used to estimate and express the extent of an ischaemic lesion but this can make comparison with other studies difficult. These include measurement of cross-sectional area (Longa *et al.*, 1989), measurement as a percentage of hemisphere (Longa *et al.*, 1989; Zhang *et al.*, 1997; Overgaard *et al.*, 1992) and quantification in absolute terms (mm^3) (Grome *et al.*, 1988) but numerical values are more meaningful for the purposes of comparison with other studies.

CONCLUSIONS

This chapter has demonstrated the establishment and validation of three stroke models. The monofilament model, both permanent and transient, were established for the first time in Edinburgh and proved to be a reproducible and readily performable model. Studies showed that there was no difference in volume of damage or oedema in the 3 models at the time point chosen. In addition, the computer based morphometric analysis for the volume of damage was established and validated for use both in future studies in this thesis and for use by other researchers in the laboratory.

CHAPTER 4

Neuroprotection Studies

INTRODUCTION

In the *Chapter 3,* it was demonstrated that there was no significant difference in the volume of tissue damage or oedema in the three rat models of MCA occlusion examined. This observation however does not necessarily imply that in each model the same pathological sequence of events occur either spatially or temporally (Zhang *et al.*, 1995). Some research groups have investigated the differences in models or the lack thereof by investigating cellular response such as the expression of particular proteins such as cell adhesion molecules (Zhang *et al.*, 1994). Another approach to investigating differences between models is the administration of pharmacological compounds known to be efficacious in reducing lesion volume. These studies may reveal differences between the models, perhaps elucidate the differences in the damage mechanisms and allow dissection of signal transduction pathways (*Chapter 1*). It is assumed that if the mechanisms that underlie the observed damage in the different models are similar then putative neuroprotectants should have similar neuroprotective efficacy when administered following ischaemia.

Modern immunosuppressant drugs, such as FK506 and cyclosporin-A (CsA,) are valuable tools for elucidating intracellular signalling systems. These compounds act at low nanomolar concentrations and display great selectivity for the immunophilins that potentially regulate the entry of gene transcription factors and participate in the ischaemic cascade to the nucleus (Snyder *et al.*, 1998a; Liu, 1993). FK506 is a neuroprotective compound used routinely

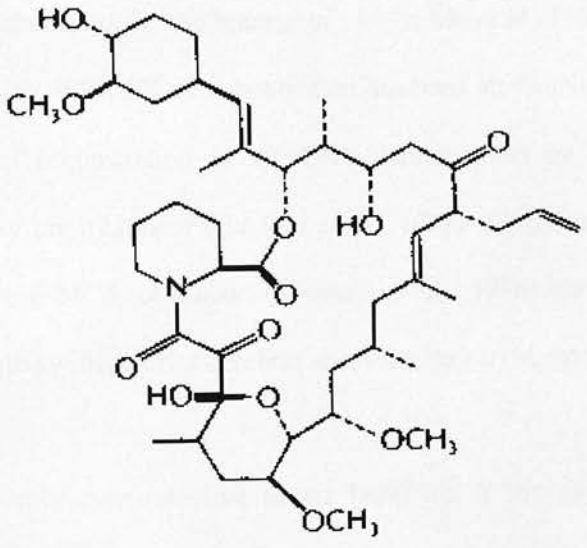
within the laboratory and has previously shown significant neuroprotection (Butcher *et al.*, 1997; Sharkey & Butcher, 1994). To this end, it is used as the routinely used as a reference compound for potential neuroprotectants under assessment. Having established the permanent and transient monofilament model of focal cerebral ischaemia within the laboratory it was of interest to investigate the neuroprotective efficacy of the compound in both models and compare it with the neuroprotection in the Endothelin-1 model and a mouse model of MCA occlusion.

FK506 (TACROLIMUS)

DISCOVERY OF FK506

Researchers at Fujisawa Pharmaceutical Co. Ltd, Tsukuba, Japan identified FK506 in 1984 through their screening program aimed at discovering compounds of microbial origin with immunosuppressant properties. The *in vitro* assay used to screen compounds measured T cell and lymphocyte proliferation in mixed lymphocyte reactions. Almost 10 000 fermentation broth samples were screened before a culture filtrate from strain # 9993 (an actinomycete) was shown to inhibit lymphocyte proliferation. Because only low levels of the active components are produced by actinomycete strains it took over a year to isolate, purify and crystallise the main active component from the fermentation broth that was named FR900506, subsequently FK506 and then Tacrolimus (Prograf®). Mycological studies identified strain # 9993 and named it *Streptomyces Tsukubaenisis* as it was isolated from a soil sample collected from Mount Tsukuba near the company's laboratories (Nishiyama *et al.*, 1995).

FK506 is a macrolide with a molecular weight of 803 and the molecular formula $C_{44}H_{69}NO_{12}$ (***Figure 17***). The compound is soluble in methanol, ethanol, ethyl acetate and chloroform and is insoluble in water and hexane. As an immunosuppressant, FK506 is 30-100 times more potent than CsA at inhibiting T cell proliferation. CsA is another well-known immunosuppressant discovered in 1976 from a fungus (*Tolypocladium inflatum*) (Borel *et al.*, 1976) and marketed by Novartis as Sandimmun®. The structure of CsA differs considerably from that of FK506 but it has a similar neuroprotective profile in a variety of ischaemia models although because of its poor BBB penetration is only effective at relatively high doses (Sharkey *et al.*, 2000; Nakai *et al.*, 1997).



FK506

Figure 17. Chemical structure of FK506 ($C_{44}H_{69}NO_{12}$).

Sharkey & Butcher (1994) first demonstrated FK506 neuroprotection following cerebral ischaemic in the Endothelin-1 and Tamura rat models of MCA occlusion (Sharkey & Butcher, 1994). FK506 is one of the few agents that protects in almost every experimental model of stroke, including transient and permanent models of focal ischaemia, in both forebrain and global ischaemia and also in a number of different species (***Table 4***) (Sharkey *et al.*, 2000). The compound is effective as a single dose given before the onset of ischaemia (e.g. 3 day pre-treatment or 30 min prior to occlusion) and has been shown to have an effective neuroprotective time window of up to 3 hrs in the rat. This time window may be greater in higher gyrencephalic species such as the cat, non-human primates and man (Sharkey *et al.*, 1994; Sharkey *et al.*, 2000; Kuroda *et al.*, 1999; Takamatsu, 1999). Neuroprotection afforded by FK506 is comparable, if not slightly greater than other neuroprotectant compounds such as MK-801 that are routinely used in studies of cerebral ischaemia (Butcher *et al.*, 1997; Stieg *et al.*, 1999; Ma *et al.*, 1998; McCulloch *et al.*, 1993). The long duration of FK506 neuroprotection has been attributed to rapid drug accumulation and preferential sequestration in the CNS demonstrated by significant neuroprotection following 3 day pre-treatment (Butcher *et al.*, 1997). FK506 also improves skilled motor deficits following MCA occlusion (Sharkey *et al.*, 1996) and ameliorates the cognitive deficits associated with anterior cerebral artery occlusion (Marston *et al.*, 2000).

FK506 is not only cytoprotective in the brain but it has also been reported to reduce ischaemia/reperfusion damage in the heart and other peripheral organs such as the liver, kidney and the skin (***Table 5***). There is also *in vitro* evidence demonstrating that FK506 is protective against excitotoxicity in culture (Tsujikawa *et al.*, 1998; Manev *et al.*, 1993; Dawson *et al.*, 1993b). The compound also has neurotrophic effects and could potentially be valuable as a therapeutic agent in other neurodegenerative disorders (Avramut, 2000).

DOXYCYCLINE

Doxycycline, a tetracycline antibiotic, was included in the neuroprotection studies firstly because it has been demonstrated to afford significant protection following an ischaemic insult. It has been shown to be neuroprotective against global brain ischaemia in the gerbil when administered 30 min post insult (Yrjanheikki *et al.*, 1998) and against transient focal ischaemia when administered prior to the onset of ischaemia (Clark *et al.*, 1997). The compound has also proved to have a beneficial effect in ischaemia/reperfusion in peripheral organs such as the liver (Smith & Gabler, 1994) and the small intestine (Smith & Gabler, 1995). The second reason for examining the neuroprotective efficacy of doxycycline is that it and other associated tetracycline derivatives have been shown to prevent microglial activation following ischaemia. As the microglia are potentially key players in the inflammatory response following an ischaemic insult, the modulation of their activation could be a potential therapeutic target (*discussed in detail in Chapter 5*).

Experimental and clinical studies indicate that doxycycline maybe beneficial in the treatment of conditions such as rheumatoid arthritis and other inflammatory diseases (Yrjanheikki *et al.*, 1998). The compound causes the suppression of oxygen radical release from polymorphonuclear neutrophils (Amin *et al.*, 1996) and inhibition of inducible nitric oxide synthase (Amin *et al.*, 1997), both which have been linked to the inflammatory response. In a study of ischaemia/reperfusion in the liver, treatment with 10 $mg.kg^{-1}$ doxycycline had a significant effect on inflammatory response inhibiting polymorphonuclear leukocyte migration, degranulation, super-oxide synthesis and cytotoxicity (Smith & Gabler, 1994). It is therefore proposed that because of its anti-inflammatory properties doxycycline may be an effective therapeutic agent, in a similar manner to FK506, by affecting multiple components of the ischaemic cascade.

MATERIALS & METHODS

Materials and methods for the neuroprotection studies are described in *Chapter 2*. Again, as in *Chapter 3*, the volume of damage and oedema are expressed as the mean ± sem in mm^3 and as difference (Δ) between ipsilateral and contralateral hemispheres as a percentage, respectively. Statistical analysis was performed using SigmaStat. For each experiment 2-way ANOVA with *post hoc* Student-Newman-Keuls test was performed.

Table 4. FK506 neuroprotection in different species & models of cerebral ischaemia (Sharkey *et al.*, 2000; McGregor *et al.*, 2000).

SPECIES	ISCHAEMIA	MODEL	REFERENCE
Rat	Focal	Surgical MCA occlusion (Tamura)	Sharkey & Butcher, 1994
		Endothelin-1 induced MCA occlusion	Butcher *et al.* 1997
			Sharkey & Butcher, 1994
			Sharkey *et al.*, 1996
		Transient monofilament	Aoyama *et al.*, 1997
			Bochelen *et al.*, 1999
			Kamiya *et al.*, 1997
			McCarter *et al.*, 2000
			Nakai *et al.*, 1997
			Toung *et al.*, 1997
		Permanent monofilament	Bochelen *et al.*, 1999*
		Permanent electrocoagulation	Bochelen *et al.*, 1999*
		Photothrombic MCA occlusion	Takamatsu *et al.*, 1999
	Forebrain	Bilateral CCA occlusion with hypotension	Drake *et al.*, 1996
		Chronic bilateral CCA occlusion	Wakita *et al.*, 1998
Mouse	Focal	Transient monofilament	McCarter *et al.*, 2000
		Transient MCA/CCA occlusion	Aronowski *et al.* 2000*
Gerbil	Global	Bilateral CCA occlusion	Ide *et al.*, 1996
			Katayama *et al.*, 1997
			Tokime *et al.*, 1996
			Yagita *et al.*, 1996
	Hemispheric	Unilateral CCA occlusion	Takana *et al.*, 1997
Monkey	Focal	Transient surgical MCA occlusion	Takamatsu *et al.*, 1999

* No observed protection MCA = middle cerebral artery CCA = common carotid artery

Table 5. FK506 protection in other organs (Sharkey *et al.*, 2000).

ORGAN	REFERENCE
Liver	Dhar *et al.*, 1996,1993,1992 Garcia-Criado *et al.*, 1997 Jin *et al.*, 1996 Kawano *et al.*, 1996,1995,1994, 1991 Kim *et al.*, 1994 Okano *et al.*, 1994 Sakr *et al.*, 1993,1991 Sawada *et al.*, 1992 Suzuki *et al.*, 1993 Wakabashi *et al.*, 1994, 1992
Heart	Morishita *et al.*, 1996 Nishinaka *et al.*, 1993
Kidney	Sakr *et al.*, 1992 Cacciarelli *et al.*, 1994 Nalesnik *et al.*, 1990
Intestine/bowel	Cicalese *et al.*, 1996 Kubes *et al.*, 1991 Sakr *et al.*, 1992
Skin	Cetinkale *et al.*, 1997

RESULTS

FK506

In both species and in the different rat models of focal cerebral ischaemia examined, the occlusion of the MCA in the vehicle treated animals resulted in a large and reproducible lesion restricted to the vascular territory of the vessel encompassing dorsal and lateral neocortex and lateral aspects of the striatum. In the rats subjected to permanent occlusion of the MCA, the mean volume of cortical and striatal infarction was 166 ± 22 mm^3 and 49 ± 1 mm^3, respectively. Animals subjected to a transient occlusion of the MCA exhibited similar cortical (151 ± 13 mm^3) and striatal (46 ± 3 mm^3) lesions. The data from the Et-1 model neuroprotection studies, included for comparison with the monofilament models, revealed a mean cortical lesion of 120 ± 15 mm^3 and striatal lesion of 35 ± 2 mm^3.

The administration of FK506 significantly reduced the volume of cortical damage in the rat permanent by 34 % compared with vehicle treated controls (***Figure 18***) and by 65 % in the transient (***Figure 19***) monofilament models. In the Et-1 model, FK506 reduced the cortical damage by 65 % (***Figure 20***). FK506 significantly reduced striatal damage by 42 % in the transient model and by 19 % in the permanent model although the latter reduction did not attain statistical significance. In the Et-1 model, FK506 reduced striatal damage by 37 % although this too was not significantly different from the vehicle treated controls. The overall neuroprotection afforded by FK506 (***Figure 21*** & ***Table 6***) in the transient model (59 % with respect to vehicle treated controls) was almost double that observed in the permanent model (34 %) but was similar to that observed in the Et-1 model (63 %) and to data previously obtained for this model in the laboratory.

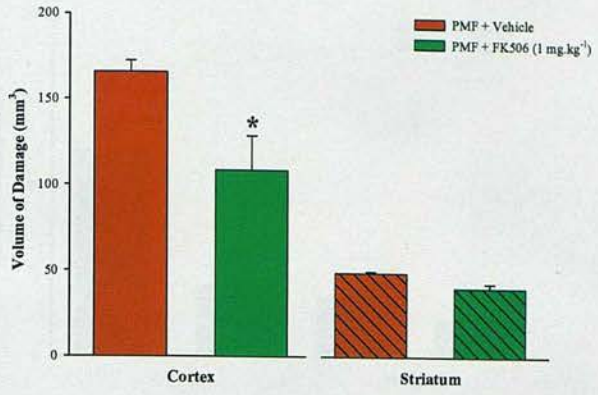


Figure 18. Neuroprotective efficacy of FK506 (1 mg.kg^{-1}, *i.v.* 5 min post-occlusion) in the permanent monofilament middle cerebral artery occlusion model (n = 12) compared with vehicle treated controls (n = 11). There is a significant reduction in the volume of damage in the cortex (34 %) in this model but not the striatum. Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test).

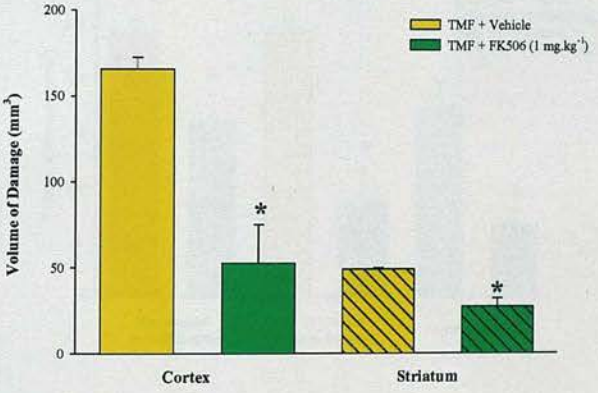


Figure 19. Neuroprotective efficacy of FK506 (1 mg.kg^{-1}, *i.v.* 5 min post-occlusion) in the transient monofilament of middle cerebral artery occlusion (n = 11) compared with vehicle treated controls (n = 8). There is a significant reduction in the volume of damage in both the cortex (65 %) and the striatum (41 %) in this model. Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test).

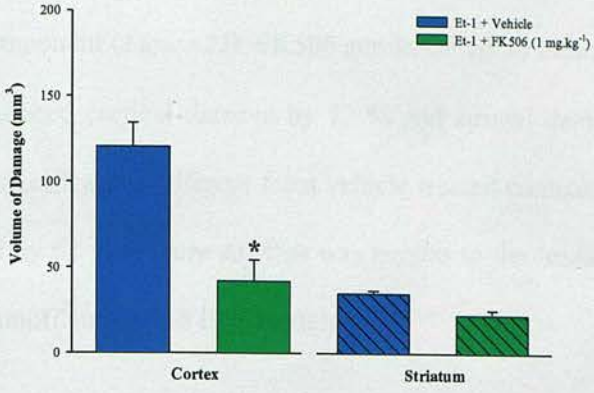


Figure 20. Neuroprotective efficacy of FK506 (1 $mg.kg^{-1}$, *i.v.* 5 min post-occlusion) in the Endothelin-1 induced middle cerebral artery occlusion (n = 8) compared with vehicle treated controls (n = 6). There is a significant reduction in the volume of damage in the cortex (65 %) but not the striatum. Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with post hoc Student-Newman-Keuls test).

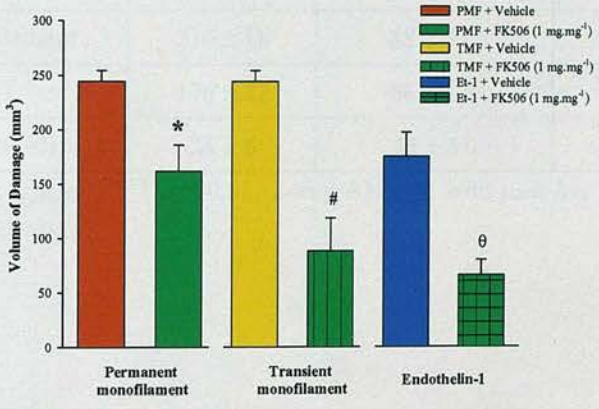


Figure 21. Comparison of the neuroprotective efficacy of FK506 (1 $mg.kg^{-1}$, *i.v.* 5 min post-occlusion) in the permanent monofilament (n = 12), transient monofilament (n = 11) and Endothelin-1 induced (n = 8) middle cerebral artery occlusion models and corresponding vehicle treated controls (n = 11, 8 and 6 respectively). Data represent mean ± sem. (*, #, θ $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test).

The ischaemic damage in the control mouse transient model was similar in distribution to that in the transient rat model displaying both a cortical (18 ± 4 mm^3) and striatal (9 ± 1 mm^3) component (***Figure 22***). FK506 administered 30 minutes prior to MCA occlusion significantly reduced cortical damage by 72 % and striatal damage by 29 % although this again was not significantly different from vehicle treated controls. FK506 treatment reduced overall damage by 61 % (***Figure 23***) that was similar to the lesion volume reduction seen in the transient monofilament and Et-1 models.

Table 6. Neuroprotective efficacy of FK506 (1 $mg.kg^{-1}$) compared with vehicle treated controls in four different models of middle cerebral artery occlusion.

MODEL	VOLUME OF DAMAGE (mm^3)		NEUROPROTECTION (%)
	VEHICLE	FK506 (1 $mg.kg^{-1}$)	
Permanent monofilament	245 ± 10	162 ± 24*	34
Transient monofilament	216 ± 18	$88 \pm 32^{\#}$	59
Endothelin-1	176 ± 22	$66 \pm 14^{\theta}$	63
Mouse transient monofilament	28 ± 6	$11 \pm 4^{\blacklozenge}$	61

Data represents mean ± sem.*, #,θ,♦ $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test)

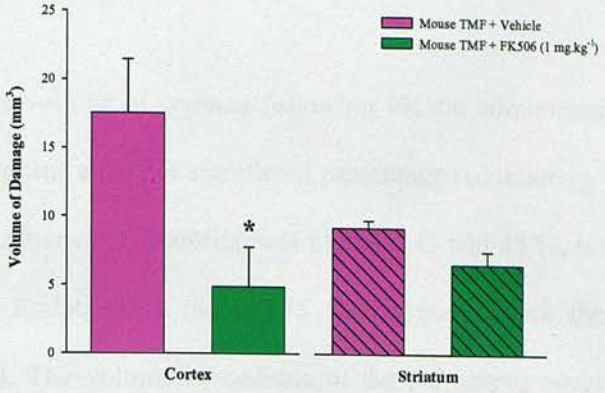


Figure 22. Neuroprotective efficacy of FK506 (1 $mg.kg^{-1}$, *i.p.* 30 min pre-occlusion) in the mouse transient middle cerebral artery occlusion (n = 6) compared with vehicle treated controls (n = 5). There is a significant reduction in the volume of damage in the cortex (72 %) but not in the striatum. Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test).

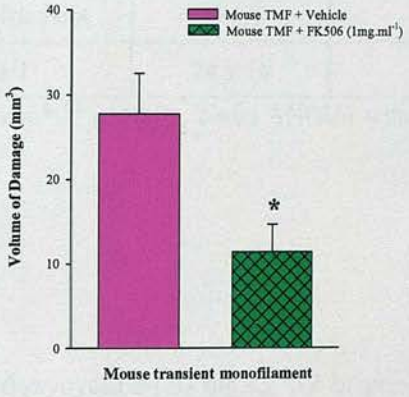


Figure 23. Comparison of the neuroprotective efficacy of FK506 (1 $mg.kg^{-1}$, i.p. 30 min pre-occlusion) in the mouse transient monofilament middle cerebral artery occlusion (n = 6) compared with vehicle treated controls (n = 5). The reduction in the lesion volume in the mouse following FK506 administration is similar to that observed in the rat permanent monofilament model. Data represent mean ± sem.(* $p < 0.05$, 2-way ANOVA with post hoc Student-Newman-Keuls test).

EFFECT OF FK506 ON OEDEMA IN THE RAT

Calculation of the volume of oedema following FK506 administration in the three models revealed that there was a similar significant percentage reduction ($p < 0.05$) in the volume in the permanent and transient monofilament models (43 and 48 %, respectively) and a greater reduction in the Endothelin-1 model (75 %) compared with the corresponding vehicle controls (***Table 7***). The volume of oedema in the permanent occlusion vehicle group was greater than that of both the transient and Et-1 models

Table 7. Comparison of the volume of oedema following FK506 (1 $mg.kg^{-1}$) administration in three models of middle cerebral artery occlusion.

MODEL	Δ HEMISPHERE VOLUMES (%)		Δ OEDEMA (%)
	VEHICLE	FK506 (1 $mg.kg^{-1}$)	
Permanent monofilament	35 ± 2	20 ± 2*	43
Transient monofilament	25 ± 3	13 ± 4$^{\#}$	48
Endothelin-1	24 ± 10	6 ± 2$^{\theta}$	75

Data represents mean ± sem.$^{*,\#,\theta}$ $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test)

DOXYCYCLINE

The administration of doxycycline (10 $mg.kg^{-1}$; 2 hr pre-treatment) (***Figure 25***) in either the permanent and transient monofilament occlusions had no effect on the volume of damage compared with saline treated controls. Replication of the Clark *et al.* experiments (1997) which involved both pre- and post-occlusion treatment (*see Chapter 2*) also showed no reduction in lesion volume (***Figure 26***) (Clark *et al.*, 1997).

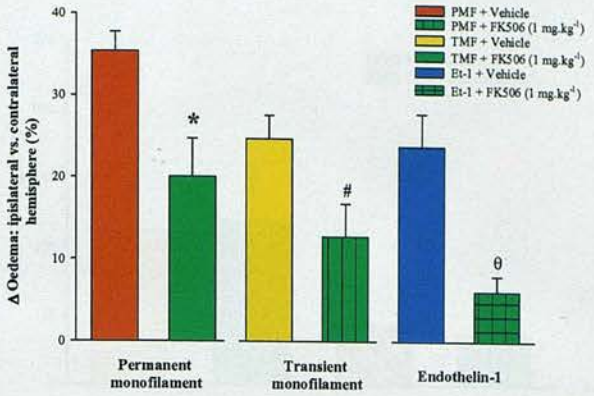


Figure 24. Comparison of the effect of FK506 (1 mg.kg^{-1}, i.v.) on the volume of oedema in permanent monofilament (n = 12), transient monofilament (n = 11) and Endothelin-1 induced (n = 8) middle cerebral artery occlusion models and corresponding vehicle treated controls (n = 11, 8 and 6 respectively). There is a significant difference between the vehicle of permanent monofilament and the other two models although it is not marked. Data represent mean ± sem. (*, #, θ $p < 0.05$, 2-way ANOVA with post hoc Student-Newman-Keuls test).

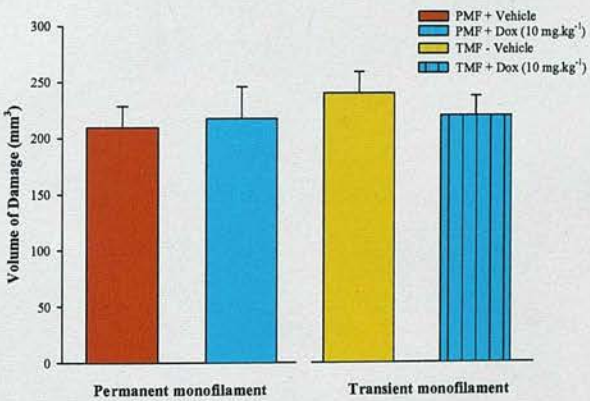


Figure 25. Comparison of the neuroprotective efficacy of doxycycline (10 mg.kg^{-1}, i.p.) in the permanent (n = 8) and transient (n = 8) monofilament middle cerebral artery occlusion models and corresponding saline treated controls (n = 6 and 9 respectively). Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with post hoc Student-Newman-Keuls test).

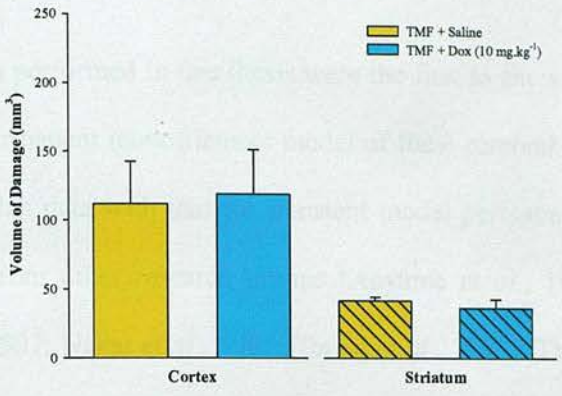


Figure 26. Neuroprotective efficacy of doxycycline (10 mg.kg^{-1}, 30 min pre-treatment *i.p*; 10 mg.kg^{-1} *i.p*, 6 x 8 hr post-occlusion) in the transient monofilament middle cerebral artery occlusion (n = 5) model and saline treated controls (n = 5). Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test).

DISCUSSION

The experiments performed in this thesis were the first to show neuroprotection afforded by FK506 in the permanent monofilament model of focal cerebral ischaemia. This has allowed comparison of this data with that for transient model permanent model data obtained here and with data from other research groups (Aoyama *et al.*, 1997; Bochelen *et al.*, 1999; Kamiya *et al.*, 1997; Nakai *et al.*, 1997; Toung *et al.*, 1997). The data from the transient rat model revealed neuroprotection in the striatum. This observation contrasts that reported by Butcher *et al.* (1997) but agrees with data produced by Bochelen *et al.* (1999) (Bochelen *et al.*, 1999; Butcher *et al.*, 1997). In the Butcher *et al.* (1997) paper, failure to see significant neuroprotection in the striatum may be associated with the statistical analysis (power effect) and increasing the experimental number could reveal a significant neuroprotection in this brain region. The studies in the mouse transient monofilament are the first show FK506 neuroprotection in this species. The data obtained for the transient and permanent models confirms the observations in *Chapter 3* that the size and distribution of the lesions the vehicle treated animals in the different rat models of MCA occlusion are similar. The neuroprotective efficacy of FK506 in the two models was however different.

The overall reduction in brain damage seen in the transient model (65 %) is similar to that reported by other groups and to that previously described by this laboratory using the Endothelin-1 model of MCA occlusion (Sharkey & Butcher, 1994; Sharkey *et al.*, 2000). The degree of neuroprotection in the rat permanent monofilament model (34 %) was smaller than that observed in the transient model and is comparable with that observed following occlusion of the MCA by electrocoagulation (Sharkey & Butcher, 1994; Takamatsu *et al.*, 1998). FK506 afforded a similar degree of cortical neuroprotection when administered either 30 minutes prior to or 60 minutes post-occlusion verifying adequate brain penetration of the

compound (Butcher *et al.*, 1997; Sharkey & Butcher, 1994). The current studies also confirm that FK506 is effective in reducing lesion volume despite route of administration (Brecht *et al.*, 1999).

The different levels of neuroprotection afforded by FK506 in the permanent and transient models may hint at the fact that cellular mechanisms that underlie tissue damage and ultimately cell death in the different models being substantially different. Zhang *et al.* (1995) demonstrated that antibodies directed at intracellular adhesion molecule (ICAM-1) were effective after transient but not permanent monofilament model, supporting the idea of a different cellular response despite the ultimate lesion observed (Zhang *et al.*, 1995). It is not inconceivable that such variations could be due to difference in the inflammatory response in the various models and again raises the question of role of reperfusion following ischaemia. The reduction observed in the permanent model suggests that FK506 has direct actions on the cells in the compromised tissue. The additional protection afforded by the compound in the transient models may result from the combination of direct neuroprotective effects coupled with anti-inflammatory actions, targeting responses associated with reperfusion. In the search for clinically effective treatments for stroke, the use of agents that interfere with the inflammatory cascade appear to confer protection only in those models that have a reperfusion component and the degree of protection (< 40 %) offered by these agents is less than that previously demonstrated for FK506 (Asahi *et al.*, 2000; Zhang *et al.*, 1995; Zhang *et al.*, 1999a). This suggests that FK506 neuroprotection may be multimechanistic i.e. modulation of the inflammatory response combined with direct cellular actions. This idea is supported by data published by Kung & Halloran (2000) contradicting the idea that immunophilins are abundant and not limiting for the inhibition of calcineurin by FK506. Inhibition of calcineurin was not complete, either *in vitro* or *in vivo* in various tissues and especially in the brain. They propose that FK506 may inhibit immune function without completely inhibiting calcineurin that may suggest inhibition of immune function is not

mediated by a general inhibition of calcineurin but by a subset of the phosphatases (Kung & Halloran, 2000).

It is important in a discussion regarding FK506 neuroprotection in cerebral ischaemia to start with a discussion of potential mechanisms that have been cited. The immunosuppressant activity of FK506 is thought to be mediated principally by the inhibiting T-cell activation and consequently the expression of cytokines such as interleukin-2 (IL-2) involved in the co-ordination of an immune response (Schreiber & Crabtree, 1992). The expression of IL-2 results from the interaction of a number of nuclear transcription factors such as nuclear factor of activated T cells (NF-AT), NF-κB and activating protein-1 (AP-1) with the IL-2 enhancer. FK506 has also been shown to inhibit lymphocyte migration *in vitro*, block the secretion of chemotactic factors and inhibit B-cell activation (Schreiber & Crabtree, 1992; Ho *et al.*, 1996).

FK506's cellular target is the FK506 binding protein FKBP12, a 12 kDa member of the ubiquitously expressed and highly conserved immunophilin family of proteins that also includes the cyclophilins, the CsA binding proteins. To date, around 20 mammalian FK506 binding proteins (FKBPs) have been identified (Hamilton & Steiner, 1998) and although the actual cellular roles of many are unknown they have been implicated in such diverse processes as gene transcription, protein secretion, intracellular Ca^{2+} release, steroid-hormone complex formation and have neurotrophic effects (Snyder *et al.*, 1998b; McGregor *et al.*, 2000). The peptidylpropyl cis-trans isomerases or immunophilins as they were termed, were first discovered in 1984 and displayed rotamase enzyme activity catalysing the isomerisation of peptide proline residues between *cis* and *trans* conformations (Fischer *et al.*, 1984). Generally referred to as immunophilins, these enzymes were originally classed by their ability to bind either cyclosporin-A (cyclophilins) or FK506 (FK506 binding proteins).

Rapamycin, another immunosuppressant compound binds to FKBP12 with greater affinity than FK506 but unlike CsA and FK506 it has no neuroprotective efficacy (Sharkey & Butcher, 1994; Snyder *et al.*, 1998a). The conversion of the *cis* and *trans* rotamers of the amide bonds by the enzymes can influence protein folding/conformation, stabilisation and docking and may be responsible for the immunosuppressive properties when bound to corresponding immunosuppressant ligand (Hamilton & Steiner, 1998; Steiner *et al.*, 1992). Interestingly, FKBP12 is upregulated in neurones of the ischaemic penumbra and in leukocytes/macrophages in the infarction, which has led to the suggestion that increased expression may help the neurones of the penumbra to survive and implicated the immunophilins in the inflammatory process after ischaemia (Kato *et al.*, 1999).

FKBP12 is the most abundantly expressed member of the FKBP proteins particularly in the brain (10 to 50-fold that observed in the immune system) and was the first member of the FKBP immunophilins to be identified (Steiner *et al.*, 1992). The protein is present in many cell types and is well conserved from plants to yeasts to humans (Snyder *et al.*, 1998a). FKBP12 participates in a number of cellular processes including transmitter release, neural NO production, nerve growth and intracellular Ca^{2+} release via the ryanodine receptor (RyR) and the inositol 1,4,5,trisphosphate receptor (IP_3) (Bultynck *et al.*, 2000). Many proteins that have rotamase activity and bind to immunophilins do not have immunosuppressive functions. Studies with 506BD, a non-natural immunophilin ligand which strongly inhibits FKBP12 rotamase activity but is not an immunosuppressant, suggest that the inhibition of the rotamase activity is in itself not responsible for the immunosuppressive actions of the compound (Bierer *et al.*, 1991; Soldin *et al.*, 1993). In addition to this, immunosuppressant compounds often act at low nanomolar concentrations and tissue levels of immunophilins are almost micromolar, only a tiny percent of rotamase activity would be inhibited (Snyder *et al.*, 1998a). The immunophilins must therefore interact with a second protein that when bound to the drug confers immunosuppressive characteristics to the complex. This protein was

identified as calcineurin (CaN), a type 2B Ca^{2+} calmodulin-activated serine/threonine protein phosphatase that is inhibited by the FKBP12/FK506 complex (Snyder *et al.*, 1998b). The co-localisation of FKBPs and CaN in most regions of the brain supports their potentially important role in the central nervous system (Steiner *et al.*, 1992; Dawson *et al.*, 1994).

Calcineurin is a heterodimer consisting of a 59 kDa A subunit with calmodulin and catalytic sites and a 19 kDa B subunit with four calcium binding sites (Morioka *et al.*, 1999; Price & Mumby, 1999; Rusnak & Mertz, 2000). First detected by Wang & Desai (1976) the enzyme was termed calcineurin on the basis of its Ca^{2+} binding properties and localisation to neuronal tissue (Kato *et al.*, 1999; Wang & Desai, 1976). CaN is widely distributed in mammalian tissue but the highest levels are detected in the brain. Different isoforms are found in other organs (Rusnak & Mertz, 2000). The immunophilins are able to bind to CaN in the absence of immunosuppressant compounds but binding is potentiated by the presence of the appropriate drug (Cardenas *et al.*, 1994). The FKBP12/FK506 complex does not impinge on the active site of CaN but binds to an adjacent site and sterically inhibits the approach and dephosphorylation of CaN substrates (Husi *et al.*, 1994). Strong evidence supporting the involvement of CaN in FK506 neuroprotection comes from observations that the immunosuppressant sirolimus (rapamycin) which binds to FKBP12 with similar affinity to FK506 but does not inhibit CaN activity and is not neuroprotective (Bochelen *et al.*, 1999; Sharkey & Butcher, 1994). It could be concluded from this evidence that immunosuppression and neuroprotection are not necessarily linked phenomena although a number of biological events that are associated with organ rejection that are suppressed by FK506 may occur in the brain following an ischaemia i.e. cytokine expression, free radical production and monocytes/leukocyte infiltration (*Chapter 1*). Another potential mechanism by which FK506 may mediate neuroprotection through the calcineurin pathway revolves around protein phosphorylation. A detrimental imbalance of protein phosphorylation can occur following an ischaemic insult as various kinases are activated (Drake *et al.*, 1998).

Morioka *et al.* (1999) suggest that calcineurin is a bi-directional enzyme in the cell death cascade, having both protective and neurotoxic actions (Morioka *et al.*, 1999).

The mechanism by which FK506 attenuates damage in cerebral ischaemia has not yet been fully elucidated although many putative mechanisms have been proposed (***Figure 27***) (Sharkey *et al.*, 2000). It is not unreasonable to suggest that FK506 neuroprotection following an ischaemic insult is the result of multi-mechanistic actions of the compound (Dumont, 2000).

Effect on physiological parameters

The direct effect of FK506 on physiological parameters of experimental animals causing reduction in ischaemic damage has been discounted. FK506 does not appear to have a direct cardiovascular effect or influence brain or core body temperature (Butcher *et al.*, 1997). It is also unlikely that the effects of FK506 are mediated through alterations in blood flow, as administration of the drug in normal rats had no noticeable effect on the regional CBF in autoradiography studies (*J. Sharkey – unpublished observations*). There is some recent evidence to suggest that FK506 may improve haemodynamics following stroke by producing a beneficial change in cerebral oxygen metabolism and oxygen extraction fraction (Takamatsu, 1999). It is however difficult to dissect if the observations are the cause or the effect related to FK506 administration. The improvement in cerebral blood flow and thus delivery of substrates required by the brain may be the consequence of the alterations in vascular reactivity and plugging by leukocytes. This could be linked to NO production and expression of adhesion molecules on the endothelial surface which both may be affected by FK506 administration (Tsujikawa *et al.*, 1998). Alternatively, the changes in cerebral oxygen metabolism and oxygen extraction fraction would reflect the direct neuroprotective effects of FK506.

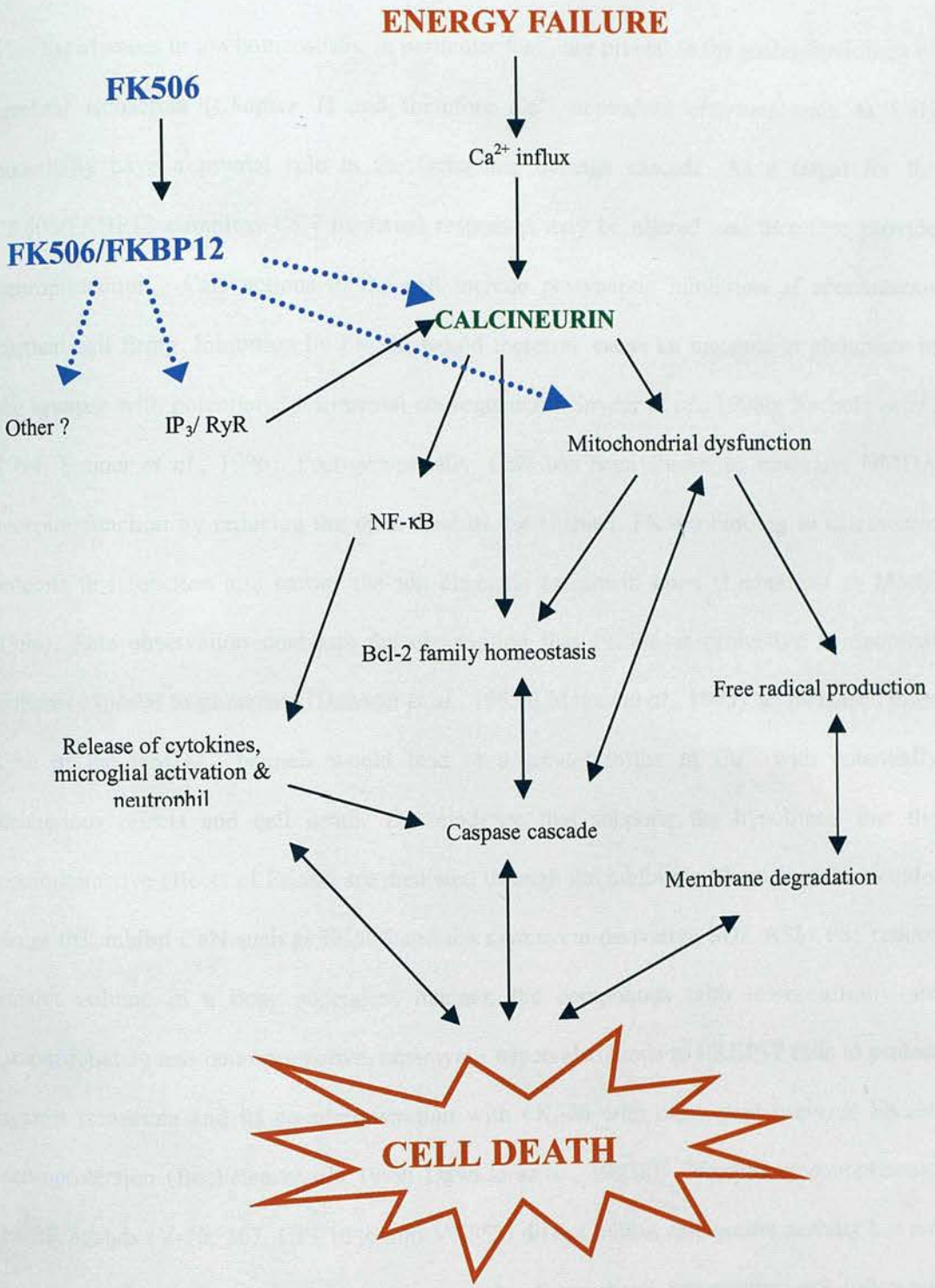


Figure 27. FK506 – proposed mechanisms of action.

Calcineurin mediated actions

The disturbances in ion homeostasis, in particular Ca^{2+}, are pivotal in the pathophysiology of cerebral ischaemia (*Chapter 1*) and therefore Ca^{2+} dependent enzymes such as CaN potentially have a pivotal role in the ischaemic damage cascade. As a target for the FK506/FKBP12 complex, CaN mediated responses may be altered and therefore provide neuroprotection. CaN actions in the cell include presynaptic inhibition of spontaneous cortical cell firing. Inhibition by FK506 would therefore cause an increase in glutamate in the synapse with potentially detrimental consequences (Snyder *et al.*, 1998b; Nichols *et al.*, 1994; Steiner *et al.*, 1996). Post-synaptically, CaN has been shown to modulate NMDA receptor function by reducing the open time of the channel. FK506 binding to calcineurin inhibits this function and causes the ion channels to remain open (Lieberman & Mody, 1994). This observation contrasts the observation that FK506 is protective in neuronal cultures exposed to glutamate (Dawson *et al.*, 1993a; Manev *et al.*, 1993), as increased open time of the NMDA channels would lead to a greater influx of Ca^{2+} with potentially deleterious effects and cell death. The evidence that supports the hypothesis that the neuroprotective effects of FK506 are mediated through the inhibition of calcineurin include: drugs that inhibit CaN such as FK506 and the ascomycin derivative SDZ ASM 981 reduce infarct volume in a dose dependent manner, the compounds with lower affinity are proportionately less neuroprotective, rapamycin which also binds to FKBP12 fails to protect against ischaemia and its co-administration with FK506 with rapamycin prevents FK506 neuroprotection (Bochelen *et al.*, 1999; Dawson *et al.*, 1993b). Non-immunosuppressant FKBP ligands (V-10, 367, GPI 1046 and VX853) do not inhibit calcineurin activity but are neuroprotective and neuroregenrative in models of peripheral neuropathy and following axotomy in the CNS, although not in cerebral ischaemia (Herdegan *et al.*, 2000; Gold *et al.*, 1998; Gold *et al.*, 1997). These observations suggest that calcineurin independent mechanism may also be involved on FK506 neuroprotection. This notion of a calcineurin

independent mechanism is also supported by the observation that CsA failed to protect the neurones of the hippocampus in a transient global model of ischaemia where FK506 was effective (Hamilton & Steiner, 1998).

The transcription factor NF-AT is an important calcineurin substrate in T cells, B cells, mast cells and other immune cells (Aramburu *et al.*, 1998). NF-AT stimulated the transcription of a number of genes such as IL-2 that are required for cell 'proliferation'. NF-AT, usually located in the cytoplasm, is unable to enter the nucleus unless dephosphylated by calcineurin. Consequently, inhibition of CaN phosphatase activity by FK506/FKBP12 complex prevents nuclear translocation of NF-AT. NF-κB although not structurally similar to NF-AT does possess functional similarities (Rao, 1994). NF-κB is a member of a family of related proteins and was first described as a nuclear factor that bound to the κ light chain in B cells. It is in fact widely expressed and regulates the expression of a variety of genes, the majority of which encode proteins that are important in immunity and inflammation (Abraham, 2000; Carroll *et al.*, 1998). NF-κB is found both in the periphery and in the CNS and in the latter it has been suggested to play a unique role in process such as neuronal plasticity, neurodegeneration and neuronal development (O'Neill & Kaltschmidt, 1997). The molecular mechanism for controlling and regulating the immediate early gene expression in the myocardium during ischaemia and reperfusion has recently been proposed to be NF-κB mediated (Squadrito *et al.*, 2000) and could therefore have a similar in the ischaemic brain.

The prototypic inducible form of NF-κB is a heterodimer composed of NF-κB1 and Rel-A subunits. Inactive NF-κB is present in the cytoplasm complexed with an inhibitory protein IκBα. NF-κB is considered to be a stress sensor molecule as its activation occurs within minutes of the stimulation and it appears that a number of stimuli can activate it including cytokines, oxidative stress and reactive oxygen species (O'Neill & Kaltschmidt, 1997). On

receiving an activating stimulus, NF-κB is released from IκB inhibition through the phosphorylation of the IκB protein with is ubiquitinated and degraded by proteosomes. NF-κB is then translocated into the nucleus, binds to the κB motif of the target gene and causes activation of several factors involved in the inflammatory response (Squadrito *et al.*, 2000). It has been shown that FK506 prevents NF-κB activation by inhibiting calcineurin that indirectly induces IκBα degradation through phosphorylation (Okamoto *et al.*, 1994). Inhibition of calcineurin blunts calcium dependent events such as NF-κB activation, TNFα gene transcription, nitric oxide synthase activation, cell degranulation, apoptosis and ICAM-1 expression (Squadrito *et al.*, 2000).

Calcium

FK506 may mediate neuroprotection by interaction with other Ca^{2+} mediated mechanisms or by directly affecting the Ca^{2+} metabolism of the cell. FKBP12 immunoprecipitates with the 300 kDa inositol 1,4,5, triphosphate receptor (IP_3R) and the 565 kDa ryanodine receptor (RyR), which are both found in the brain and regulate the release of Ca^{2+} into the cytosol from internal stores such as the ER (Hamilton & Steiner, 1998; Jayaraman *et al.*, 1992). FK506 disrupts FKBP12 interaction with RyR receptor increasing the probability of opening and the mean open time of the channel and renders the receptor 'leaky' to Ca^{2+} ions (Snyder *et al.*, 1998a). FKBP12 interacts directly with the IP_3 thereby modulating the flux through the channel with FK506 having different effect in different tissues and it is thought that FK506-FKBP12 may fine tune the Ca^{2+} fluxes through the channel (Bultynck *et al.*, 2000). FKBP12 does not appear to be required for channel function but it does appear to influence receptor stability (Brillantes *et al.*, 1994). FK506 neuroprotection may therefore be explained by the prevention of cellular Ca^{2+} overload during reperfusion period (Nakai *et al.*, 1997).

Mitochondrial function

The mechanisms that underlie the direct neuroprotective effect of FK506 are unclear although it has recently been proposed that the drug acts through the stabilisation of the mitochondria (Friberg *et al.*, 1998). Mitochondrial failure caused by the lack of oxygen and glucose results in the pronounced decrease in cellular energy (ATP) levels during an ischaemic insult (*Chapter 1*). The mitochondria act as a calcium sinks during ischaemia, sequestering cytosolic Ca^{2+} until a critical level is reached whereupon respiration ceases, free radicals are produced and the contents of the cells are released into the cytosol (Fiskum, 1985). When mitochondria are exposed to high concentrations of Ca^{2+} ions a large proteinaceous megachannel also named the mitochondrial transition pore opens in the inner mitochondria. It is the opening of this pore that allows the passage of solutes up to 1500 kDa to flow across the membrane into the cytoplasm (*Chapter 1*). Evidence to support the formation of the MTP and its role in the ischaemic cascade comes from observations that CsA prevents formation of the MTP by blocking translocation of cyclophilin-D to the inner membrane thereby decreasing sensitivity to Ca^{2+} ions. CsA, as mentioned previously, is neuroprotective but at higher concentrations because of its poor BBB penetration (Friberg et al, 1998). Results from *in vitro* experiments suggest that FK506 is less effective that CsA as an inhibitor of MTP-related Ca^{2+} release. FK506 mechanisms preventing mitochondrial dysfunction are different and at present are unknown but it is suggested that FK506 protects mitochondria by indirectly affecting Ca^{2+} uptake (Nakai *et al.*, 1997). The MTP is thought to be an early event in apoptosis in some cells and possibly the trigger for cell death in ischaemia-reperfusion damage although recent data suggests the brain mitochondria may be highly resistant to MTP formation and that pore opening is not crucial to the release of apoptotic factors such as cytochrome *c* (Berman *et al.*, 2000; Ouyang *et al.*, 1999; Perez-Pinzon *et al.*, 1999). The restoration of substrates that occurs with reperfusion allows a preliminary partial recovery of mitochondrial respiratory function and ATP synthesis (Almeida *et al.*, 1995). This is however followed by a subsequent secondary mitochondrial degeneration during the first few hours of reperfusion that is associated with the generation

of free radicals that can damage cellular components (Chan, 1996). FK506 has been shown to prevent secondary mitochondrial dysfunction following transient ischaemia in the rat (Nakai *et al.*, 1997).

It is plausible that the differences observed in the permanent and transient models with respect to the efficacy of FK506 may be due to an improvement of post-ischaemic mitochondrial function associated with reperfusion. Studies by Folbergrova *et al.* (1995) showed that following 2 hrs of MCA occlusion there was an initial partial recovery of the bioenergetic state of the focal and penumbral tissues (1 hr) with a secondary deterioration at a later point (4 hrs). The secondary bioenergetic failure was thought to be due to delayed mitochondrial dysfunction (Folbergrova *et al.*, 1995). The notion of secondary mitochondrial failure is supported by the observation that phenyl-*N*-tert-butyl nitrone, a free radical spin trap, ameliorated damage even when administered 1-3 hrs after recirculation (Zhao, 1994). Drugs and antibodies that show beneficial effects in terms of damage reduction if given 1-3 hrs post-reperfusion are either free radical scavengers or curb the inflammatory response. Both free radicals and inflammation can be associated with mitochondrial dysfunction and are linked to one another (Nakai *et al.*, 1997). Nakai *et al.* (1997) demonstrated that FK506 ameliorated the secondary mitochondrial dysfunction and improved post-ischaemic respiratory rates *in vitro*, which could explain the difference in the degree of neuroprotection observed in these studies (Nakai *et al.*, 1997).

Suppression of apoptosis

As discussed in *Chapter 1*, the contribution of apoptosis to ischaemic cell death is a much-debated topic and consequently compounds that may affect the apoptotic mechanisms are of interest. The opening of the MTP in the mitochondria following an ischaemic insult releases apoptotic effectors such as cytochrome *c*, apoptosis inducing factor and Ca^{2+} which

are all capable of initiating the cell death cascade (Cassarino *et al.*, 1997; Susin *et al.*, 1997). FK506 could potentially influence apoptosis through a number of calcineurin-mediated events linked to mitochondrial dysfunction. The anti-apoptotic bcl-2 protein is closely associated with the mitochondria and under ischaemic conditions can increase the amount of Ca^{2+} that can be sequestered by the mitochondria (Murphy *et al.*, 1996). Bcl-2 not only improves the capacity of the mitochondria to handle calcium overload but also to protect from the adverse effects of calcium accumulation thereby influence mitochondrial vulnerability (Shibasaki & McKeon, 1995). Bcl-2 forms a complex with calcineurin that inhibits its phosphatase activity and may explain the anti-apoptotic actions of bcl-2. The binding of FK506/FKBP12 complex to calcineurin could potentially influence cell survival in two ways. By binding to calcineurin there is sustained inhibition of the enzyme and additionally the bcl-2 protein is released and may interact with and inhibit the actions of the pro-apoptotic proteins such as BAX (Yang *et al.*, 1995) via the cytochrome *c*/caspase pathway and via interactions with other apoptotic mechanisms. BAD, a pro-apoptotic member of the Bcl-2 family, is also a substrate for calcineurin and its dephosphorylation enhances heterodimerisation with anti-apoptotic proteins and leads to apoptosis (Rusnak & Mertz, 2000). There are also a number of observations from both neuronal and non-neuronal cells that may suggest FK506 affects apoptotic cell death either directly or indirectly by mechanism that do not involve calcineurin. These mechanisms include modulation of the MAP kinase pathways (Winter *et al.*, 1998) and ceramide induced apoptosis (Herr *et al.*, 1999).

Nitric oxide production

One of the most cited mechanisms of FK506 mediated neuroprotection is the attenuation of NO-related free radical production via the inhibition of nitric oxide synthase (Ide *et al.*, 1996; Tanaka *et al.*, 1997; Tokime *et al.*, 1996; Yagita *et al.*, 1996). As described previously in *Chapter 1*, NO combines with other superoxide free radicals forming reactive species that cause cellular damage through peroxidation of lipids, oxidation of sulphydryl groups, perturbations of membrane permeability and enzyme function. FK506 inhibits NMDA-stimulated NOS production and blocked NMDA-induced neurotoxicity in cultured neurones (Manev *et al.*, 1993; Dawson *et al.*, 1993a). In these experiments, FK506 is thought to provide neuroprotection, in part, by preventing the dephosphorylation and the activation of neuronal NOS by calcineurin (Dawson *et al.*, 1993a). *In vivo* data however contradict these results. FK506 failed to prevent excitotoxic damage resulting from intracerebral microinjections of NMDA or quinolate suggesting that protection is mediated by other by other mechanisms (Butcher *et al.*, 1997; Globus *et al.*, 1995). Administration of a selective neuronal NOS inhibitor following an insult had a less pronounced effect than that of FK506 suggesting that FK506 may ameliorate ischaemic damage through other mechanisms (Nakai *et al.*, 1997). The contribution of NO to the pathology of stoke is a confused area of research and consequently the efficacy of FK506 may be embroiled in this confusion. Recently however, it has been shown that FK506 did not alter NO production during ischaemia and early reperfusion measured by microdialysis suggesting that neuroprotection is not mediated through inhibition of NO production (Toung *et al.*, 1999). The interaction of FK506 with NO synthases following an ischaemic insult may potentially be the mechanism for neuroprotection although there are somewhat confused results regarding this mechanism. Toung *et al.* (1999) demonstrated that FK506 administration had little effect on the total NOS activity following ischaemia when measures indirectly by assaying for arginine-to-citrulline conversion. They speculated that the isoforms measured in the 6 hours

following MCA occlusion were principally neuronal and endothelial but did not account for the inducible/immunological isoform iNOS (Toung *et al.*, 1999). It is feasible that FK506 may prevent the activation of iNOS and preventing the maturation of the lesion as it may be activated beyond the first few hours of reperfusion. This hypothesis would fit with observations that FK506 suppresses the production of iNOS in cultured macrophages (Conde *et al.*, 1995). With respect to the permanent and the transient models, the differences seen in the response may reflect the involvement of iNOS if it is assumed that the inflammatory response in the permanent model is less pronounced than in the transient model. If this were to be the case, the mechanisms of damage that occurred in the two models would be a consequence of different pathways but result in similar lesion sizes.

Anti-inflammatory Actions

In peripheral organs such as the heart, liver, gut and skin and within *in vitro* experiments FK506 has been shown to have effects on components of the inflammatory cascade. The drug attenuates cytokine expression, inhibits neutrophil infiltration into tissue (Nishinaka *et al.*, 1993; Kubes & Ward, 2000; Sakuma *et al.*, 2000), reduces free radical production from monocytes and PMNLs and alters macrophage/microglial response to noxious stimuli (Hortelano *et al.*, 1999; Andersson *et al.*, 1992a; Keicho *et al.*, 1991; Matsuda & Baba, 1998; Wakita *et al.*, 1998). Ischaemia experiments in the heart suggest that FK506 affects a number of components of the inflammatory response. It blocks the early activation of NF-κB, suppresses ICAM-1 gene activation, reduces leukocyte accumulation and protects against myocardial damage (Squadrito *et al.*, 2000). FK506 has also been shown to inhibit neutrophil infiltration into ischaemic tissue in the CNS. Tsujikawa *et al.* (1998) employed a novel approach to investigate the effect of FK506 of leukocyte accumulation. The procedure was non-invasive and looked at the effect of FK506 on leukocyte dynamics following retinal ischaemia (Tsujikawa *et al.*, 1998). The conclusions drawn from the experiments suggested

that FK506 had an inhibitory effect on leukocyte rolling and subsequent accumulation resulting in reduction of damage. The alterations in leukocyte dynamics could be explained in a number of ways as the recruitment of these cells is a multistep process but this could also provide insight into the mechanism of FK506 neuroprotection. Reduced rolling of the leukocytes may result from a reduction in the cell adhesion molecules on the PMNLs or the endothelium whose expression could be FK506 sensitive. FK506 could be acting by affecting NO production and altering vasoactivity or suppressing the release of cytokines that attract the leukocytes and the expression of cell adhesion molecules (Tsujikawa *et al.*, 1998).

FK506 also affects other elements of the immune system such as the microglia within the CNS. *In vivo* experiments demonstrated that FK506 protected white matter in the brain by the attenuation of glial cell response (Wakita *et al.*, 1998). The effect of FK506 on microglia has also been demonstrated *in vitro* where it re-transformed LPS activated microglia to their ramified state, down regulated NO production and IL-6 synthesis, proposed to occur through NF-κB-DNA binding inhibition (Herdegan *et al.*, 2000). FK506 has been shown to affect expression of inflammatory signalling molecules in other scenarios. In a model of chronic joint inflammation, FK506 was shown to significantly reduce the expression of IL-6 and NO thereby reduce joint swelling (Fuseler *et al.*, 2000). FK506 also reduced an increase in tumour necrosis factor α (TNFα) following 6-hydroxydopamine lesioning in rats. *In vitro*, FK506 potently inhibited TNFα and IL-1β production by human peripheral blood mononuclear cells, as well as suppressing adhesion molecules on vascular endothelial cells (Sakuma *et al.*, 2000). This observation supports a report that demonstrated that FK506 inhibited neutrophil chemokinesis and suggested it was calcineurin mediated adhesion molecule expression that was the target for the drug (Hendey *et al.*, 1992). The conclusions from experiments with FK506 and components to the inflammatory response tend to suggest the compound affects multiple components of the immune response.

In all three models, the reduction in the volume of oedema was similar. It is difficult to establish whether the reduction in oedema is purely the consequence of the reduction of damage or if the actions of FK506 in some way alter the development of oedema associated with ischaemic damage. Oedema measurements for the mouse model were not performed, as the mouse monofilament model is not central to the thesis but included as an interesting comparison in terms of neuroprotection afforded by FK506.

Doxycycline has been reported to afford neuroprotection in rat MCA occlusion models (Clark *et al.*, 1997; Yrjanheikki *et al.*, 1999), in global ischaemia in the gerbil (Yrjanheikki *et al.*, 1998), in rabbit models of spinal cord ischaemia and arterial air embolism (Clark *et al.*, 1994b; Reasoner *et al.*, 1997) and in peripheral organs like the liver and the intestine (Smith & Gabler, 1994; Smith & Gabler, 1995). The neuroprotection studies performed with doxycycline revealed that there was no difference in the volume of damage in either the transient or the permanent model with drug treatment. These observations contradict the observations mentioned above. It is possible that the doxycycline dose administered in the initial studies (10 $mg.kg^{-1}$) was not sufficient to mediate neuroprotection. This treatment regime was based on protocols used previously in peripheral tissues (Smith & Gabler, 1994; Smith & Gabler, 1995) given as a single dose administered 2 hrs pre-ischaemia to allow brain penetration in order to assess this treatment regime in the rat. In previous studies of global and focal ischaemia, multiple doses of doxycycline were been administered in the gerbil and rat (Yrjanheikki *et al.*, 1998; Yrjanheikki *et al.*, 1999) and perhaps this dosing regime was required for the drug to enter the brain. However, the experiments with multiple does of doxycycline (pre- and post insult) based on the Clark *et al.* protocol (1997) also showed no neuroprotection (Clark *et al.*, 1997). It is possible that multiple dosing 8 hours post insult is beyond the therapeutic window for neuroprotection. The reasons for the failure to show neuroprotection in these studies are however essentially unclear.

CONCLUSIONS

FK506 is neuroprotective in both the transient and permanent monofilament models of focal cerebral ischaemia, the Endothelin-1 model and additionally in the mouse monofilament. The difference in the neuroprotective efficacy of FK506 in the two different rat models suggests that there are different pathological processes between the two models and it is proposed that the differences may be due to a difference in the post-ischaemic inflammatory response. The final volume of damage may not be different but the processes involved in the development of the damage respond differently to the drug. One potential cellular candidate identified as a possible target for FK506, is the microglial cells and their response to an ischaemic insult and drug administration may explain the different degrees of FK506 neuroprotection observed. Whilst there have been several reports of the anti-ischaemic efficacy for doxycycline in three species and in a variety of tissues, this protection was not demonstrated in these studies. A pharmacokinetic explanation is unlikely but cannot be ruled out.

CHAPTER 5

Microglia

INTRODUCTION

The data presented so far have established that the permanent and transient models of focal cerebral ischaemia, whilst producing a similar volume of damage, respond to differently to neuroprotectants such as FK506. The observations in *Chapter 4* with respect to the percentage reduction in damage in different models of MCA occlusion raised the question of the possibility of different cellular responses following insult that lead to infarction. Three major cells types within the brain parenchyma are thought to be involved in the inflammatory processes that occur in the brain following an insult: endothelial cells, astrocytes and microglia and there has been much investigation into the roles of each of these cell types in CNS injury such as cerebral ischaemia (Gebicke-Haerter *et al.*, 1995; Giulian *et al.*, 1993; Stoll *et al.*, 1998). Infiltrating cells such as the leukocytes begin to enter the damaged parenchyma around 12 hrs post insult and monocytes/macrophages enter at an even later time point. Both cell types are present in the brain at times beyond the therapeutic window of neuroprotection perhaps making them a less likely candidate for acute tissue damage (Hallenbeck, 1996; Garcia *et al.*, 1994).

The microglia, as stated previously, respond rapidly to insults to the brain and parallel the onset of earlier neuronal damage (Kato & Walz, 2000; Gehrmann *et al.*, 1992; Kreutzberg, 1996). In this thesis, the focus of investigation is the microglial cells primarily because of their rapid response to injury that has been reported by others (Kato & Walz, 2000) making

them an attractive therapeutic target. Astrocytes are generally regarded as having a beneficial role (Kato *et al.*, 1994; Raivich *et al.*, 1999) (*see Chapter 1*) and were not studied in preference for the microglia, those role is potentially deleterious. PMNL contribution to ischaemic damage is thought to be a more delayed event in relation to the response of the endogenous cells and therefore not of interest in this instance (Hallenbeck, 1996; Garcia *et al.*, 1994). Closely linked to the leukocytes and their infiltration are the endothelial cells (site of adhesion of rolling leukocytes) and thus outwith area of direct interest. If inflammation is detrimental to the brain tissue and to be the target of pharmacological agents, the earlier the intervention in the pathophysiological cascade the more beneficial effects a particular treatment may have.

Microglial cells are distributed throughout the brain although more common in grey than white matter, they constitute 5-12 % of the cells in the CNS (20 % of the glial population) and are considered to be the resident macrophages of the brain (Gehrmann *et al.*, 1992; Zielasek & Hartung, 1996). Within the brain, there are a number of populations of microglia. The majority are found in the parenchyma but there are other populations associated with the vasculature, the choroid plexus and the meninges that are distinguished by slight differences in morphology, expression of different cell surface antigens and location (Perry & Gordon, 1988; Perry *et al.*, 1994). Recent data suggests that microglia from different regions of the brain have different densities and susceptibility to noxious stimuli (Kim *et al.*, 2000). This view has been supported by the observation that microglial gene expression of inflammatory mediators depends on their localisation in the brain and on interactions with other neural cell types (Ren *et al.*, 1999). The perivascular cells are considered by some investigators not to be true microglia but a population of perivascular monocytes that are not part of the brain parenchyma and function as professional phagocytes (Graeber & Streit, 1990; Streit *et al.*, 1999).

Microglial cells were first described by Nissl in 1891 but were defined as a distinct cell type within the nervous system, with characteristic morphology and specialised staining features that differentiated them from the other glial cells and the neurones, by Rio-Hortega in 1932 (Gonzalez-Scarano & Baltuch, 1999; Moore & Thanos, 1996). The cellular origin of the microglia has been debated for many years and the prevailing view currently is that they arise from monocytes derived from the bone marrow. The cells migrate into the developing brain before the formation of the BBB where they are maintained as a pool with a low turnover during adulthood (Davis *et al.*, 1994; Kreutzberg, 1996; Perry & Gordon, 1988; Thomas, 1992). A unique property of the microglia is their ability to proliferate thereby maintaining a constant population size in the adult brain (Davis *et al.*, 1994). There is however another view suggesting that microglia are renewed by invading peripheral blood borne macrophages (Lawson *et al.*, 1992).

MICROGLIAL FORMS

Morphologically, microglia have been described as amoeboid, ramified and reactive (***Figure 28***) and are considered to be different forms of a single cell type (Thomas, 1992; Streit *et al.*, 1999). Amoeboid microglia are seen in developing brain tissue and have a broad flat morphology with pseudopodia and are the precursors to the ramified form. During embryonic development there is considerable cell death which has lead to the idea that, within the CNS, the microglia are involved in the removal of cellular debris following this natural cell death that is usually apoptotic in nature (Moore & Thanos, 1996). Ramified (or resting) microglia are seen in the normal adult brain. They have a small cell body (5-10 μm) with several thin branching processes and are considered to be downregulated under normal physiological conditions, in keeping with the idea of the immune privilege status of the brain and reflecting the specialised microenvironment of the CNS (Kreutzberg, 1996). Reactive

microglia appear in adult tissue following injury or infection and are characterised by oval or rod shaped morphology with no ramified process, their ablilty to migrate to the site of damage or infection and their phagocytic capabilities (Thomas, 1992). Some authors distinguish between activated and reactive microglia when the microglial cells become upregulated. Activated microglia appear as swollen ramified cells and are characterised by a larger cell body and shorter stouter processes and are not necessarily phagocytic (Davis *et al.*, 1994). The activation of microglia does not always result in full blown phagocytes (Thomas, 1992; Kreutzberg, 1996). Streit *et al.* (1999) also describe a hyper-ramified state of the microglia that they suggest signifies the beginning of the microglial hypertrophy in acute pathological events and which is also seen in ageing brains and may contribute to the formation of senile plaques (Streit *et al.*, 1999).

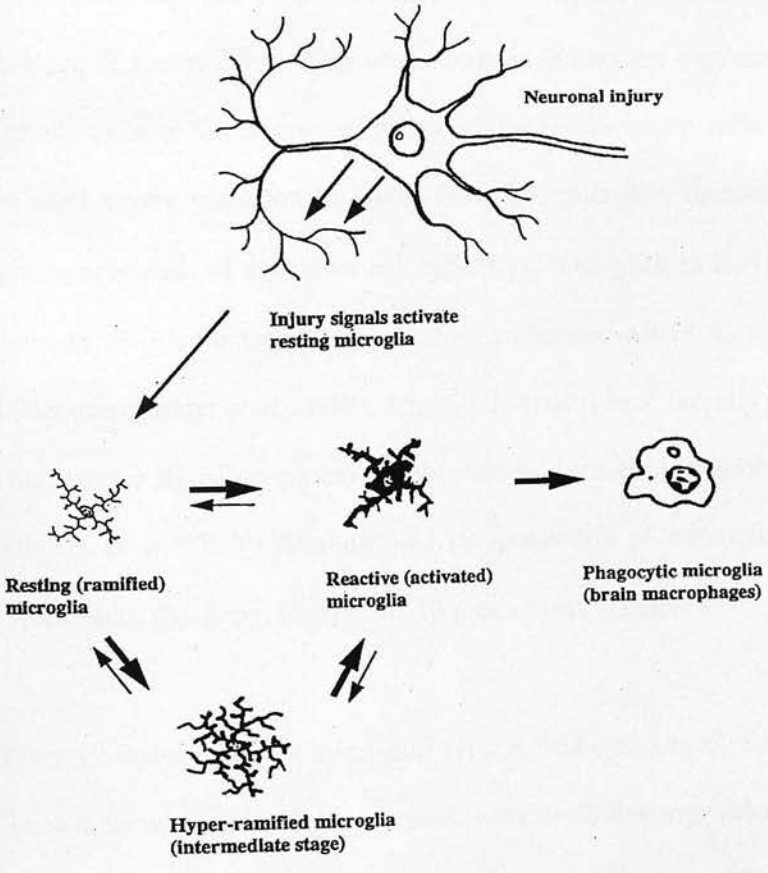


Figure 28. Morphological forms of microglia (Streit *et al.*, 1999).

MICROGLIAL ACTIVATION

The precise activation mechanisms of microglial cells are, as yet, not fully elucidated (Nakamura *et al.*, 1999). The microglia are under strict control *in vivo* and the signals that activate microglia are unclear, although there are many potential candidates. As suggested earlier, chemical messengers released from injured neurones may cause activation although a massive insult to the brain may activate the cells. The expression of immediate early genes in both the neurones and microglial could suggest that they are directly affected by the ischaemic insult (Streit, 1993). It has been proposed that the reduction in blood flow may directly activate the microglia following studies in the hippocampus where the strongest microglial activation was seen in areas with the densest capillary network suggesting reduced flow in well supplied areas cause more serious alterations than in less well supplied zones (Abraham & Lazar, 2000). This observation is in keeping with the idea of a graded response of microglia to the severity of injury, as this region would suffer the most damage due to the most severe reduction in blood flow. The microglia themselves may actually produce potent activators of their own cell type. Cytokines such as IL-1β and TNFα may cause activation in disease situations including ischaemia where they are known to be produced (Gebicke-Haerter *et al.*, 1996). Streit *et al.* (2000) have recently provided evidence that IL-6 serves as a signalling molecule that induces microglial proliferation (Streit *et al.*, 2000). Mabuchi *et al.* (2000) demonstrated the possibility of microglia contributing the gradual expansion of the infarct through IL-1β production (Mabuchi *et al.*, 2000).

Another potential activator of the microglial cells is the high extracellular potassium levels that are known to accompany both neuronal necrosis following ischaemia and also in situations were spreading depressions occur (Nedergaard & Hansen, 1988). *In vitro* studies show the microglia possess potassium channels that make them sensitive to depolarising

events (Zhang *et al.*, 1997). Other microglial mitogens such as macrophage colony stimulating factor (M-CSF), chemokines, neuropeptides and neurotransmitters may also play a role in activation (Perry *et al.*, 1994; Thomas, 1992; Kreutzberg, 1996). Fractalkines, a recently discovered class of chemokines that are cleaved from the cell membrane during ischaemia, have become a potential candidate as an activator of microglia following ischaemia (Chapman *et al.*, 2000; Zujovic *et al.*, 2000). *In vitro* experiments with gangliosides, glycoshingolipid-containing sialic residues that are particularly rich in neuronal membranes and may regulate the function of several inflammatory cells suggest that they can activate microglia (Pyo *et al.*, 1999).

ASSESSING MICROGLIAL ACTIVATION

Activated microglia are distinguished not only by changes in morphology (hypertrophy and proliferation) but also by marked changes in phenotype including upregulation of existing (CR3 complement receptor) and *de novo* synthesis of surface molecules such as major histocompatibilty complex (MHC) molecules (antigen presenting proteins on immune cells) (Streit, 1993; Lehrmann *et al.*, 1997). Monoclonal antibodies against the cell surface antigens, such as OX-42 against the CR3 receptor, have allowed the definition of microglial activation in terms of immunophenotypic changes (Thomas, 1992). OX-42 is a reliable, widely used marker of the microglia and its staining is sensitive enough to show even slight changes in microglial activation (Abraham & Lazar, 2000). The complement receptors such as CR3 are on cells that participate in immune reactions and are found on both resting, activated and phagocytic microglia and it is the intensity of staining combined with the morphological assessment that allows the various forms to be distinguished from one another. OX-42 was chosen as the marker for microglial activation in this thesis primarily because it had previously been used within the laboratory but also because it is widely used by other researchers. (In this thesis, no attempts to distinguish the sub-populations of

microglia found in the brain were made). Other antibodies such as those raised against the ED1, MHC and lectins antigens and phosphotyrosine have also been used to investigate microglia (***Table 8***) (Kato *et al.*, 1996; Korematsu *et al.*, 1994; Thomas, 1992; Barron, 1995). Using a panel of antibodies to known microglial/macrophage antigens, Lehrmann *et al.* (1997) also demonstrated the differential progression and demarcation of the infarct and the penumbral zones by the immunophenotype of the cells and confirmed the suggestion that there is a spatiotemporal pattern of microglial activation that accompanies neuronal injury and that also reflects severity of insult (Lehrmann *et al.*, 1997). Until recently microglial activation has primarily been assessed by histological methods (immunoregulatory molecule expression) which is a suitable method for studying the extent of a lesion but if considered in conjunction with the changes in gene transcription it may be possible to elucidate the functional mechanisms of the activation process (Streit *et al.*, 1999; Gebicke-Haerter *et al.*, 1996; Spleiss *et al.*, 1998). Gene expression (mRNA levels) of the substances known to be released by the microglia such as the inflammatory cytokines may fluctuate in an injury specific manner (Streit *et al.*, 1999). Experiments that link changes in microglial morphology to the changes in gene expression may help to elucidate the functional sequealae of activation. These molecular mechanisms may provide targets for intervention that may reduce damage (Raivich *et al.*, 1999).

Table 8. Histological markers for microglial cells (Nakajima & Kohsaka, 1998).

Marker	Amoeboid Microglia	Ramified Microglia	Reactive Microglia
Antibody			
OX-42	+ +	+	+ +
Mac-1	+ +	+	+ +
OX-18	+	-	+
OX-6	+	-	+
Ed-1	+ +	-	+ +
F4/80	+ +	+	+ +
CD4	+	+	+ +
Vimentin	+ +	-	+ +
Lectin			
B_4 isolectin	+ +	+ +	+ +
Agglutinin-120	+ +	+ +	+ +
Receptor			
Fc receptor	+ +	+	+ +
Acetylated LDL receptor	+ +	-	+ +
Silver impregnation	+ +	+ +	+ +
Enzyme			
Non-specific esterase	+ +	-	+ +
Thiamine pyrophosphatase	+ +	+ +	+ +

OX-42 – CR-3 complement receptor
OX-18 – MHC class I antigen
OX-6 – MHC class II antigen
ED-1 – macrophage cytosol protein

MICROGLIA IN CEREBRAL ISCHAEMIA

Ischaemic cell death is accompanied by, and generally preceded by, the activation of the microglia. Activation tends to exceed the area of the ischaemic lesion and differing morphological responses have been shown suggesting different roles of the cells in the different regions (i.e. core vs. remote ipsilateral cortex) (Schroeter *et al.*, 1999). These cells therefore can be viewed as an early, sensitive and reliable marker of threatening neuronal damage (Gehrmann *et al.*, 1992; Rupalla *et al.*, 1998). The functional changes in neurones

that occur within seconds of an ischaemic insult may initiate the changes in the microglia. Ábráham & Lázár (2000) propose that the changes in the neurones which may be reversible if counter-acted makes the early microglial reaction more important than previously thought (Abraham & Lazar, 2000).

Morioka *et al.* (1991) conducted the first examination of the microglial response to ischaemia in 1991 in a rat transient forebrain model using antibodies against the microglial/macrophage marker *Griffonia simplicifolia* B_4 isolectin and showed an early activation of the cells (20 min post insult) (Morioka *et al.*, 1991). This study was followed by an examination of microglial activation following permanent MCA occlusion in the rat. Although cell death in global and focal ischaemia are known to be different, the microglial response was found to be similar, displaying early activation and progressive expression of immunomolecules that peaked at 2 days (Morioka *et al.*, 1993). The second Morioka (1993) study concentrated only on the neocortex and thalamic areas, with no mention of the microglial activation state in the striatum. In a transient model of MCA occlusion, Lehrmann *et al.* (1997) noted progressive changes in the activation of the microglia and claimed to be able to define the penumbral tissue of both the cortex and striatum. An acute striatal response (within hours) was distinguished from the later protracted reaction in the cortex (24-72 hrs) (Lehrmann *et al.*, 1997). Zhang *et al.* (1997) examined the temporal profile of microglial response following transient (2 hr) MCA occlusion and also showed the paralleled progression of microglial response and progression of neuronal damage similar to that seen in the permanent model. In this study, there were differences in the microglia in mildly and severely damaged tissue. Microglia were absent in rapidly necrotic tissue such as the core of the infarct with ramified cells found at the ischaemic boundary (Zhang *et al.*, 1997). In the centre of the ischaemic insult, where infarction develops, it is difficult for any brain cells of any type to survive and it therefore presumed that the lack of staining in these regions is due to the failure of the microglia to survive (Kato *et al.*, 1996). The chronological course and

severity of neuronal cell death in the ischaemic area are reported to be different in the permanent and transient models of focal ischaemia and it is suggested that an earlier microglial reaction in the transient model may be due to differences in the severity of the ischaemic insult between the two models (Korematsu *et al.*, 1994). The difference between the endogenous macrophages of the brain and invading cells is difficult to determine because they express similar cell surface antigens and therefore a potential cavaet to microglia investigations. Studies have proved that there is no early change in number of macrophages suggesting that the macrophages that appear must be of microglial origin (Korematsu *et al.*, 1994). Recruitment of blood borne monocytes is however a delayed process occurring beyond 24 hrs of ischaemia/reperfusion (Zhang *et al.*, 1994) with massive infiltration after 2-3 days (Kato *et al.*, 1996).

MICROGLIA IN CULTURE

Many studies have been conducted using isolated microglia in order to dissect the mechanisms that lead to activation, to analyse secreted products and to ultimately assess the contribution of the cells to either neurodegeneration or neuroregeneration (Sudo *et al.*, 1998; Ren *et al.*, 1999; Nakamura *et al.*, 1999; Petrova *et al.*, 1999; Sawada *et al.*, 1989; Daval *et al.*, 1995; Dawson *et al.*, 1993b; Chao *et al.*, 1992; McMillian *et al.*, 1994). *In vivo*, it is difficult to attribute direct functional meaning to observations made from tissue sections, whereas *in vitro* it has been virtually impossible to establish the relevance of observations in a culture dish and relate them to the whole organ (Streit, 1993). Investigations on cultured microglia, including slice preparations, are useful to test hypotheses and additionally can help to reduce the number of animals used experimentally (Gebicke-Haerter *et al.*, 1996). It is important to set up methods for assessing morphology and *in situ* localisation of microglial secretory products in the whole brain but the use of simplified systems as far as is feasibly possible can help to construct arguments before moving to a more complex scenario. In this

manner it may be possible to build a complete picture in a stepwise and clearly identify components of a multifaceted cascade such as that of microglial activation.

MICROGLIA & FK506

FK506 as described before (*Chapter 4*) ameliorates neuronal necrosis in both global and focal models of ischaemia and in a number of species. Wakita *et al.* (1998) demonstrated that FK506 prevents microglial activation in the white matter of animals subjected to chronic cerebral ischaemia (bilateral CCA occlusion) and attenuated lesion formation (Wakita *et al.*, 1998). Immunosuppressive drugs including FK506 have been shown to downregulate mRNA expressions of proteins such as CD4 molecules that are expressed by the microglia (Sawada *et al.*, 1992). *In vitro* studies by Herdegen's group in Kiel have shown that FK506 prevents the activation of microglia by LPS and causes LPS activated microglia to become ramified again (Mielke *et al.*, 1999). FK506 exerts moderate inhibition of iNOS expression in LPS/IFNγ activated peritoneal macrophages and prevents apoptosis through inhibition of caspase 3 and an alteration in the levels of bcl-2 family proteins (Hortelano *et al.*, 1999). Experiments on cultures of macrophages from the periphery showed that FK506 inhibited the production of NO by affecting nitric oxide synthase (Conde *et al.*, 1995; Wakita *et al.*, 1998), suppressed IL-2 production and inhibited inflammatory cytokine production (Sakuma *et al.*, 2000; Wakita *et al.*, 1998). These mechanisms may explain the neuroprotective capabilities of FK506 in the brain through similar actions. It is appreciated that peripheral macrophages are not identical to microglia of the CNS but it does illustrated the sensitivity of these cells, sharing a common lineage with microglia, to the administration of FK506 and may hint at a possible mechanism of neuroprotection.

An additional set of experiments have been included in this chapter examining the effect of hypoxia on pure neuronal cultures and neurones cultures with different glial populations. This is a continuation of work already performed in the laboratory and is directly related to the assessment of the inflammatory response to cerebral ischaemia.

MATERIALS & METHODS

NEUROPROTECTANTS & MICROGLIAL ACTIVATION

Animals were subject to MCA occlusion as described in *Chapter 2*. FK506 and its vehicle were administered as previously described. In an additional experiment to complement the doxycycline neuroprotection studies, doxycycline (10 $mg.kg^{-1}$, Sigma) was administered to following both transient and permanent MCA occlusion to investigate its effect on microglial activation. A further experiment with doxycycline was also performed to assess microglial activation. Animals received 45 $mg.kg^{-1}$ of the compound 12 hrs prior to MCA occlusion and were sacrificed at 3 hrs post insult.

MICROGLIAL IMMUNOSTAINING

For the immunostaining of microglial cells, animals were deeply anaesthetised by *i.p* injection of Euthatal and transcardially perfused as previously described with heparinised PBS followed by periodate-lysine-paraformaldehyde (PLP, *Appendix 1*). Brains were removed and placed in PLP/20 % sucrose for approximately 3 hrs at room temperature before being transferred to 20 % sucrose solution overnight. Free floating cryostat sections (30 μm) were placed in PBS, washed and incubated in 1 % hydrogen peroxide for 15 min. Ipsilateral and contralateral hemispheres of the brain were marked prior to cryostat

sectioning. Sections were washed 3 times in PBS-5 % polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma) and incubated with 4 % normal horse serum (Vectastain Elite ABC Mouse Immunoglobulin G (IgG) Kit, Vector Laboratories) diluted in PBS-Tween 20. Following an overnight incubation at 4°C in the primary antibody OX-42 (1:2 000 dilution; Mouse IgG2a – MCA275G, Serotec) the sections were washed 3 times in PBS and incubated for 1 hr at room temperature in horse anti-mouse biotin labelled secondary antibody (1:250 dilution; Vector). Sections were washed and incubated for 1 hr in Avidin-Biotin Complex (ABC) Elite (Vector) at room temperature. Sections were washed twice in PBS. Antibody staining visualised with diaminobenzidine (DAB, Vector). Sections were cleared in Histoclear and mounted in DPX (BDH Chemicals Ltd, UK).

MICROGLIAL CULTURES

The protocol for microglial cultures was adapted from Levison & McCarthy (Levison & McCarthy, 1989). Cortices were removed from 12-16 one day old (range P0-P2) rat pup brains. The meninges were removed and the tissue chopped finely with a sterile razor blade. The dissection stage was carried out in Hank's Balanced Salt Solution (HBSS, GibcoRPL, Life Technologies) without calcium, magnesium or phenol red. The chopped tissue was placed in HBSS containing 0.25 % filter sterilised trypsin and 0.025 % filter sterilised deoxyribonuclease 1 (DNase, Sigma) and incubated at 37°C for 15-20 min. An equal volume of complete Basal Medium Eagles (BME) culture medium (*Appendix 2*) containing 0.025 % DNase was added and the suspension triturated through a fine bore pipette in order to disrupt the tissue. Following every 9 or 10 triturations, the tissue was allowed to settle and the cells in suspension removed. The collected suspension was centrifuged at 500 x (gravity) g for 10 min (Mistral 2000, MSE) and the pellet resuspended in an appropriate volume of complete BME culture medium. The suspension was then filtered through sterile nylon

gauze (125 μm, Lockertex) to remove clumped cells then plated out into 75 cm^2 filter top flasks (NUNC™ Δ Surface, Life Technologies) at approximately 1 x 10^7 cells per flask. Flasks were kept in the CO_2 incubator (5 % CO_2, 37°C and 99 % relative humidity, LEEC) with the medium changed after 1 day and then every 3 days until the cells were confluent (7-10 days). The medium, conditioned by the cells in the mixed cultures, was retained and filter sterilised for use in subsequent experiments.

Microglia were isolated from the mixed cultures after approximately 14 days in culture. Microglia were detached by shaking the flasks in a temperature controlled orbital shaker (Grant SS40-2, Grant Instruments) at 37°C at 200 rpm for 90 min. The medium was removed and centrifuged at 500 x g for 5 min and the pellet was resuspended in glial conditioned medium. The microglia were placed in 75 cm^2 NUNC filter top flasks at a density of 5 x 10^6 cells in glial conditioned medium for 24 hrs before treatment.

LPS STIMULATION

Microglial cultures were challenged with LPS (1 $\mu g.ml^{-1}$) for both 3 and 24 hrs and compared with saline treated control cultures. The activation state of the cells was assessed by morphology and photographed (Axiovert 135 Microscope, Zeiss; Contax 167MT camera; Kodak Tmax 100 Pro). At the appropriate time points, protein or total RNA was extracted from the cells for use in reverse transcription polymerase chain reaction (RT-PCR) and immunoblotting, respectively (*Chapter 6*).

EFFECT OF FK506 ON LPS STIMULATION

FK506 (5 μM) and vehicle respectively were added to the LPS stimulated cells immediately following LPS administration. Again morphology was noted and protein or RNA extracted for analysis at 3 and 24 hrs.

IN VITRO 'ISCHAEMIC' INSULT

Primary cultures of rat cerebrocortical neurones were prepared from the cortices of embryonic day 17 (E17) Sprague Dawley rats. Cortices were dissociated for 12 min in HBSS containing 0.09 % trypsin and 500 IU/ml DNase, triturated in HBSS containing 500 IU/ml DNase and plated out at 1600 cells/mm^2 in Neuronal Plating Medium (containing 5 % heat-inactivated horse serum, 5 % heat-inactivated foetal bovine serum) onto poly-D-lysine coated 15 mm wells. After 24 hrs, the medium was replaced with B27 medium and thereafter partly refreshed twice weekly. On day 5, the cultures were treated with 5 mM cytosine β-D-arabino-furanoside (ara-c, Sigma), which as a mitotic agent resulted in a pure neuronal culture. Cultures were used for experimentation at 11-15 days *in vitro.* To establish neurone and astrocyte or microglia only cultures, neurones were plated over the respective glial culture.

To establish astrocytic monolayers, cultures were prepared as described for microglial cultures described above. Mixed culture flasks were shaken in the orbital shaker for 90 min to detach the microglia (collected and plated) and then shaken further overnight. The remaining adherent population was then trypsinised and replated onto 24 well plates (5.6×10^4 cells per well) and returned to the incubator for 7 days until confluent. Neurones were then plated over the astroglial layer and used for experimentation after a further 11-15

days. Neuronal/microglia only cultures were established from the collected medium from the first shake. Microglia were plated as described in the microglial culture section above. Neurones were plated over the microglia at both 1 day and 7 days post initial microglial plating as the cells did not appear to become confluent and comparison was made between 1 and 7 days. The natural mixed glial population was obtained by not treating the original E17 primary cultures with ara-c that were plated into 24 well plates and used for experimentation 11-15 days. Glial content of these cultures was estimated previously to be 12-20 % by immunostaining.

Cultures were subjected to anoxia (0 % O_2, 5 % CO_2, 94 % N_2, 37°C) for 30, 60, 90, 180, 360 and 1440 min in an anoxic chamber (Don Whitely Scientific MACS VA) followed by the appropriate recovery time in the CO_2 incubator at 37°C until assessed by MAP-2, fluorescein isothiocyanate (FITC) immunostaining at 24 hrs for neurones and mixed cultures and lactate dehydrogenase (LDH) cytotoxicity assay (CytoTox 96®, Promega) staining for the microglial and astrocyte only cultures. Briefly, cells were washed with PBS and fixed with PLP (*Appendix 1*), washed and permabilised with 0.25 % triton in PBS. Cells in the plates were blocked in 10 % BSA in PBS for 20-30 min and incubated in primary antibody (anti-MAP-2, Sigma) diluted 1:1000 in 3 % BSA for 2 hrs at room temperature. Cells were washed and incubated with the FITC linked secondary antibody dilutes 3 μg.ml-1 in 3 % bovine serum albumin (BSA) in PBS for 1 hr at room temperature in a dark chamber. Plates were washed with PBS and the signal read using a Fluoroskan Ascent plate read using an excitation wavelength of 485 nm and emission wavelength of 520 nm. LDH assay was performed according to manufacturer's instructions (Technical Bulletin) and the absorbance read at 490 nM in the Dynex Technologies plate reader.

RESULTS

IN VIVO MICROGLIAL ACTIVATION

The staining of the microglia with the OX-42 monoclonal antibody took several months to establish. In the initial experiments, staining on slide-mounted paraformaldehyde fixed 20 μm sections was not consistent and it was difficult to visualise the microglia. The experiments with 30 μm free floating PLP perfusion fixed sections provided a better-defined stain of the cells. By marking the contralateral hemisphere of the brain before sectioning, it was possible to correctly orient the brain when the sections were slide mounted. It was also discovered that the post-perfusion fixation in 20 % sucrose/PLP had to be limited to 3 hrs where upon the brains were removed and placed in 20 % sucrose in PBS solution overnight. Exclusion of the primary OX-42 antibody confirmed the specificity of the OX-42 staining for microglia.

Once established, the visualisation of the microglial cells showed activated microglia with enlarged cell bodies and retracted process (***Figure 29***) in both the cortex and striatum of the ipsilateral hemisphere following a 3 hr MCA occlusion. Staining of sections from animals that had undergone anaesthesia only was diffuse (***Figure 30***) confirming that microglial activation did not occur with routine anaesthesia and that the change in morphology and therefore staining in the occlusion animals was indeed a consequence of the ischaemic insult. The staining of both the cortex and striatum of the contralateral hemisphere was diffuse indicating no activation of the cells and therefore valid as a control (***Figure 31*** & ***Figure 32***). In one of the three sham animals, the morphology of the cells was not completely ramified like that of the anaesthetic only control sections nor did they show the activated morphology seen in lesioned tissue (*upper left panel in* ***Figure 32***).

FK506 vehicle had no effect on microglial activation. In the FK506 treated animals, the morphology of the microglia was similar to that of the sham and the anaesthetic only groups suggesting that the drug may prevent microglial activation (***Figure 33***). In the striatum, similar observations regarding the activation state of the microglia were made (***Figure 34***). The administration of FK506 appeared to make no difference to the morphology of the cells in the contralateral hemisphere, which was not unexpected, as they did not display an activated morphology.

The administration of doxycycline at both doses (10 and 45 $mg.kg^{-1}$) had no effect on the activation state of the microglia which was consistent with the neuroprotection studies where no reduction in lesion volume was seen (*data not shown*).

Following these initial positive results, subsequent experiments looking at microglial activation using the OX-42 antibody produced spurious results. Staining was not consistent suggesting that the activation of the cells was not consistent making assessment of drug effects virtually uninterpretable. Permanent occlusion experiments (3 hrs) were repeated and studies extended to the transient model (2 hr occlusion plus 1 hr reperfusion). In an experiment with LPS, known to activate microglia, the results were also inconsistent. Fresh primary, secondary antibodies and DAB were used in order to improve the staining with little success. The possibility of the monofilament having not occluded the MCA was ruled out in each permanent model experiment by the verification of placement on removal of the brain amd in the transient model by Evans Blue staining. No other variables had been changed in the experimental procedure.

IN VITRO MICROGLIAL ACTIVATION

Initial studies examined the effects of culture conditions on microglial morphology. Media conditioned by the cells of the primary mixed cultures proved to be better than freshly prepared media and was therefore routinely retained during the culturing process for the experiments that followed. NUNC plates provided a better surface (in comparison with Falcon plates and poly-L-lysine coated dishes) for the cells to adhere to and ramify. The plating density of the cells was also varied and optimum density in 75 cm^2 flasks was approximately 5 x 10^6 cells. Initially, microglia were allowed to settle and ramify for 72 hrs once dissociated prior to LPS stimulation. This time was shortened to 24 hrs as the cells looked healthier and more ramified at the earlier time point.

Although cells in culture did not resemble those *in vivo*, processes could clearly be seen in the resting cells (***Figure 35***) and upon LPS stimulation there was a noticeable change in the morphology (***Figure 36***). FK506 administration appeared to affect the activation of the cells assessed subjectively under the microscope. OX-42 immunostaining and Griess reagent were used to assess the activation state of the cells and to detect changes following both LPS stimulation and FK506 administration. The OX-42 antibody used the cells *in vivo* did not stain the cells *in vitro*. Griess reagent used to detect NO produced by cells in culture (Pyo *et al.*, 1999) as a marker of microglial activation was also unsuccessful.

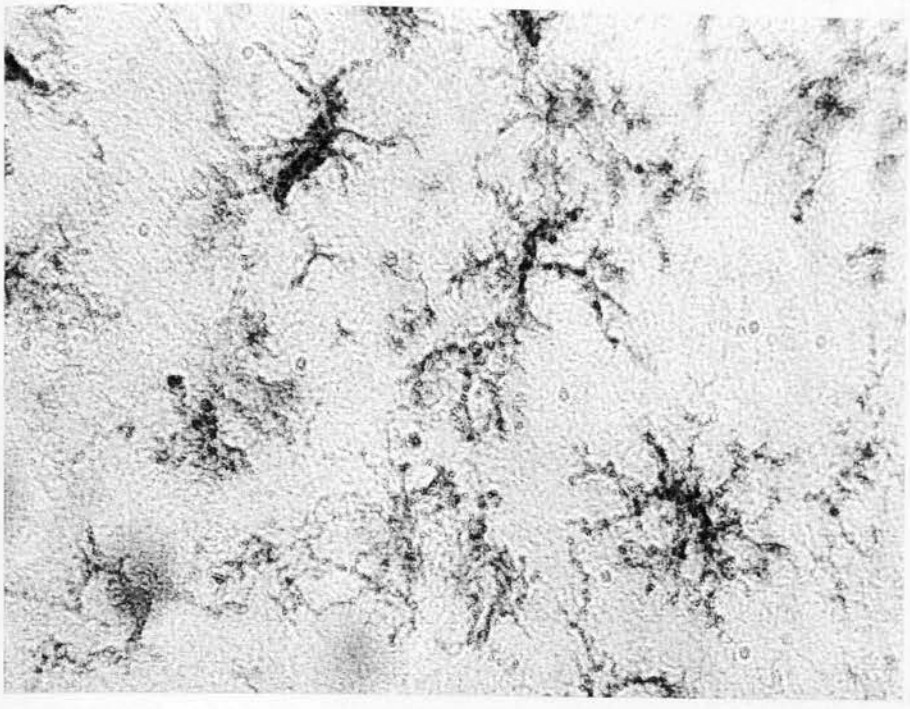


Figure 29. Typical activated microglia visualised with OX-42 (40 x magnification) following 3 hrs monofilament middle cerebral artery occlusion showing larger cell bodies and stouter retracted processes.

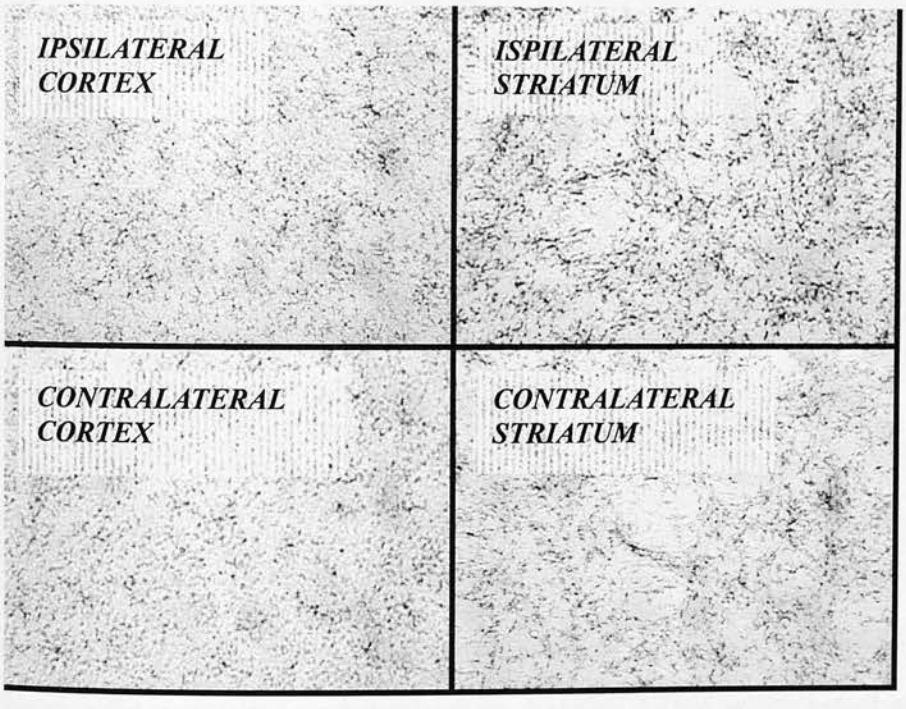


Figure 30. Comparison of ipsilateral and contralateral cortex and striatum (10 x magnification) in a control rat brain (anaesthetic only) killed at 3 hrs post induction of anaesthesia.

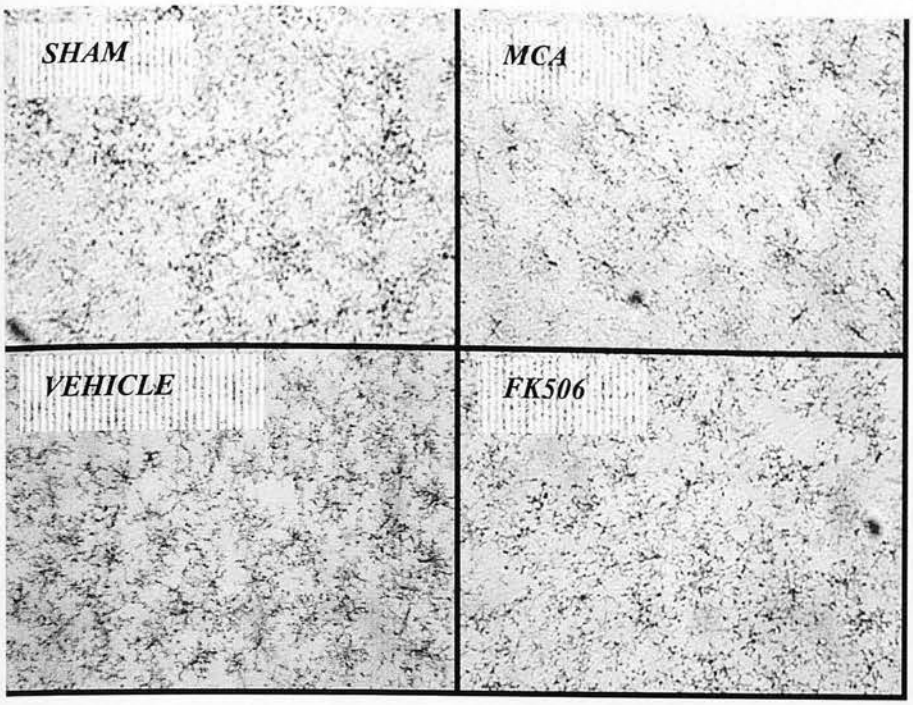


Figure 31. Comparison of microglial activation following a 3 hr permanent monofilament MCA occlusion in the contralateral cortex of a sham, occlusion, FK506 vehicle treated and an FK506 treated animal (10 x magnification).

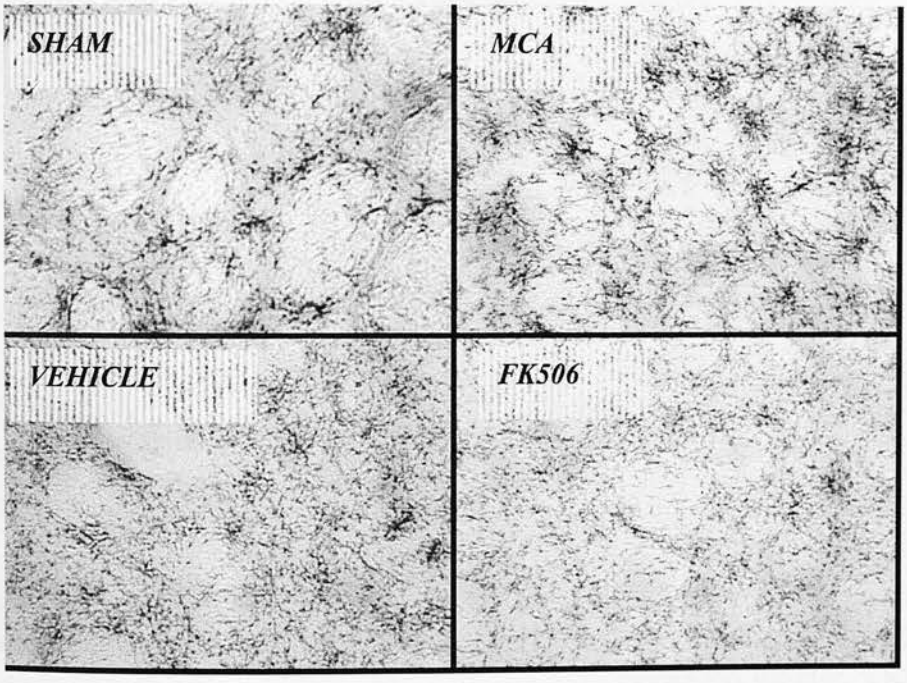


Figure 32. Comparison of microglial activation following a 3 hr permanent monofilament MCA occlusion in the contralateral striatum of a sham, occlusion, FK506 vehicle treated and an FK506 treated animal (10 x magnification)

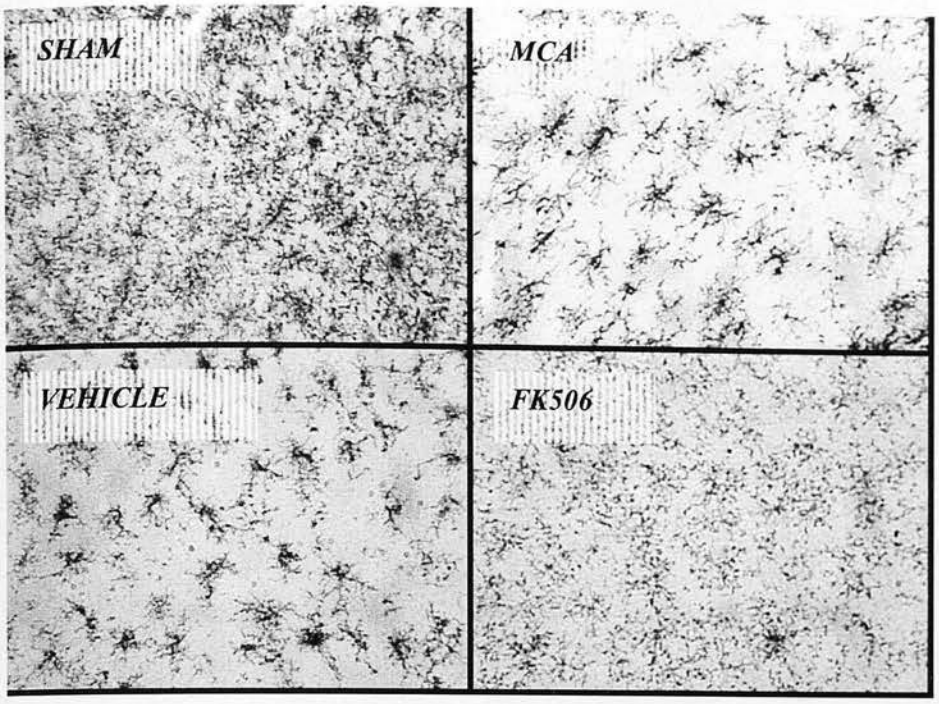


Figure 33. Comparison of microglial activation following a 3 hr permanent monofilament MCA occlusion in the ipsilateral cortex of sham, occlusion, FK506 vehicle treated and an FK506 treated occlusion (10 x magnification).

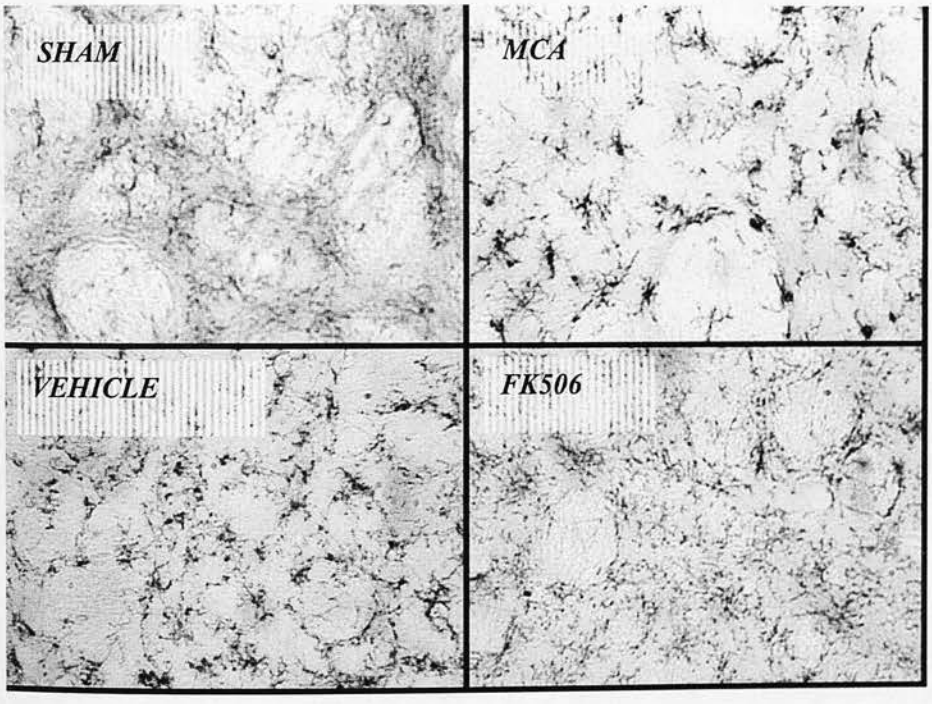


Figure 34. Comparison of microglial activation following a 3 hr permanent monofilament MCA occlusion in the ipsilateral striatum of an anaesthetic control, occlusion, FK506 vehicle treated and an FK506 treated occlusion (10 x magnification).

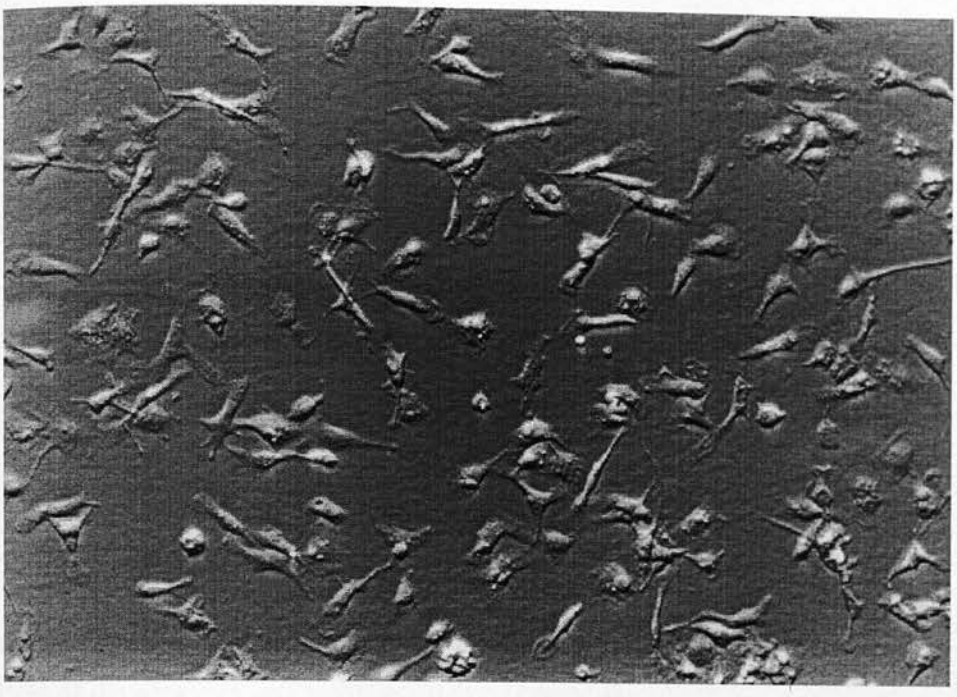


Figure 35. *In vitro* ramified microglial cells (20 x magnification).

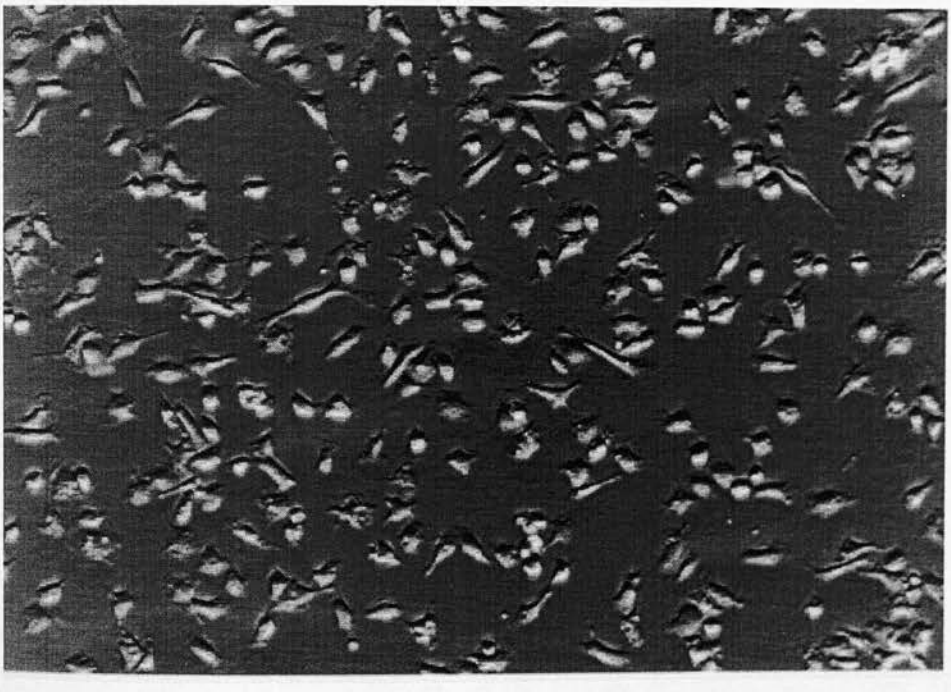


Figure 36. Activated microglia in culture following 3 hrs LPS stimulation (20 x magnification)

Exposure of pure neuronal cultures to OGD caused cell death within 30 min. The presence of glia (12-20 %) co-cultured with the neurones significantly prolonged the survival time of the neurones, reaching the same extent of cell death as the pure neuronal cultures by 90 min. At 30 min approximately 50 % of the neurones were still viable. Analysis of neurones co-cultured with pure astrocytes evaluated at 7 days post plating with the neurones showed a similar profile to the natural mixed population (***Figure 37***). Exposure of neurones co-cultured with microglia, evaluated 7 day post plating with the neurone, showed a similar viability profile to the mixed glial population and the pure astrocyte co-culture with approximately 55 % of the neurones still viable at 30 min (***Figure 38***). Interestingly, neurones cultured with pure microglia and assessed 1 day post plating with the neurones survived longer (30 min) than those assessed 7 days post plating. Following 30 min of OGD, 70 % of the neurones were still viable and 50-55 % at 60 min. By 90 min the extent of cell death in these cultures was no different to the pure neuronal and natural mixed glial cell co-cultures (***Figure 39***).

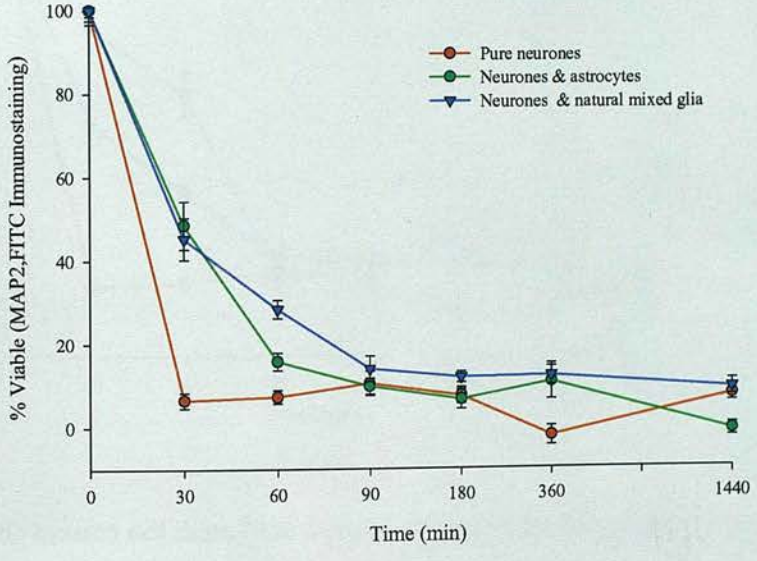


Figure 37. OGD induced cell death (0 % O_2/0.5 mM glucose for time points between 30 amd 1440 min assessed at 24 hrs post insult) in cultured rat cortical neurones, neurones cultured with astrocytes and neurones cultured with natural mixed glia (2-12 %).

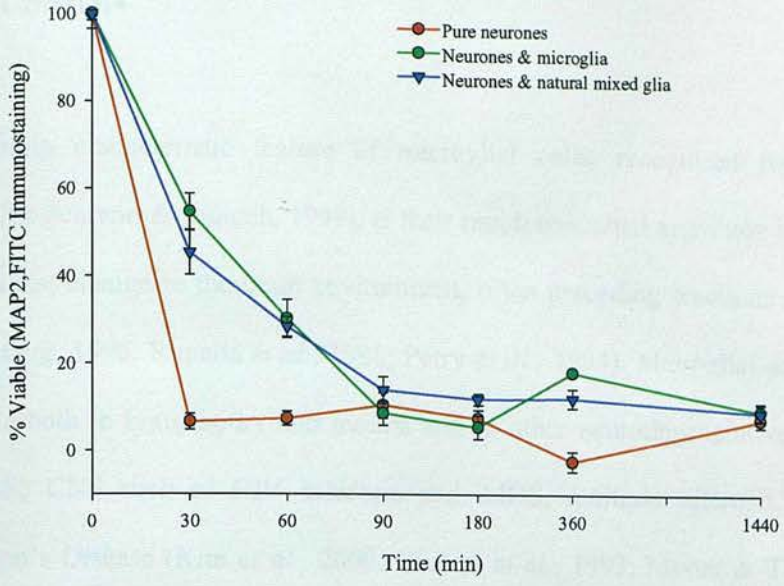


Figure 38. OGD induced cell death (0 % O_2/0.5 mM glucose for time points between 30 amd 1440 min assessed at 24 hrs post insult) in cultured rat cortical neurones, neurones cultured with 7 day old microglia and neurones cultured with natural mixed glia (2-12 %).

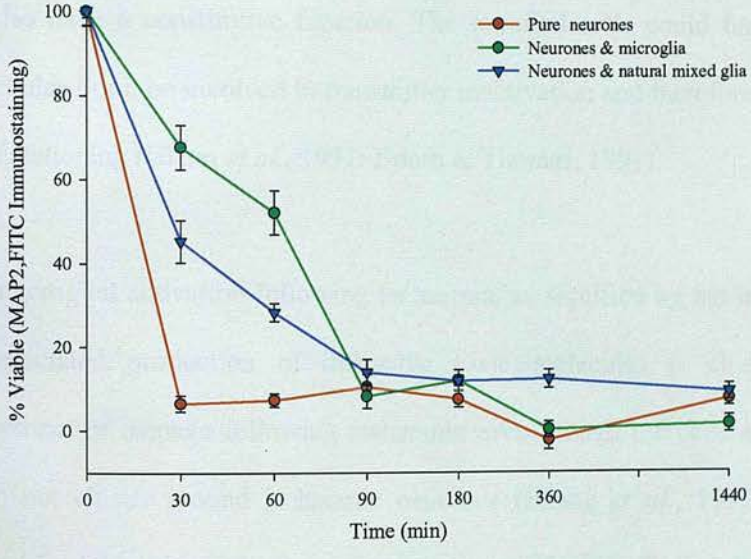


Figure 39. OGD induced cell death (0 % O_2/0.5 mM glucose for time points between 30 amd 1440 min assessed at 24 hrs post insult) in cultured rat cortical neurones, neurones cultured with 1 day old microglia and neurones cultured with natural mixed glia (2-12 %).

DISCUSSION

A defining characteristic feature of microglial cells, recognised for over a century (Gonzalez-Scarano & Baltuch, 1999), is their rapid, concerted activation in response to even the smallest change in the brain environment, often preceding reactions of other cell types (Kreutzberg, 1996; Rupalla *et al.*, 1998; Perry *et al.*, 1994). Microglial activation is seen in response both to brain injury and trauma and in other neurodegenerative diseases that can affect the CNS such as HIV infection and AIDS, multiple sclerosis, Alzheimer's and Parkinson's Disease (Kim *et al.*, 2000; Giulian *et al.*, 1993; Moore & Thanos, 1996). The activation of the microglia in response to injury becomes more pronounced and widespread with more severe insults (Raivich *et al.*, 1999) although the cells appear to exhibit a non-specific upregulation in response to different types of damage (Davis *et al.*, 1994; Kreutzberg, 1996). Although much attention has been focused on the microglia as the macrophages of the brain following injury or infection it has also been suggested that they may also have a constitutive function. The ramified cells could function in cleaning the intracellular fluid, be involved in transmitter inactivation and therefore have a role in normal brain functioning (Glenn *et al.*, 1991; Booth & Thomas, 1991).

Early microglial activation following ischaemia, as signified by the morphological changes and associated production of diffusible toxic molecules is closely linked with the development of damage following ischaemia even though the cells are not yet phagocytic and do not cluster around ischaemic neurones (Zhang *et al.*, 1997). Kim *et al.* (2000) provided *in vivo* evidence for the neurotoxic nature of microglia in a study where the greatest cell death was seen in regions of the brain containing the greatest number of microglia in response to intracerebral administration of LPS (Kim *et al.*, 2000). Microglia have also been implicated in secondary selective neuronal degeneration following ischaemia in regions (e.g.

the thalamus and substantia nigra) distal from those involved in the primary lesion (Rupalla *et al.*, 1998) and also in delayed neuronal damage through the production neurotoxins released by the reactive cells (Giulian & Vaca, 1993; Lees, 1993). The cellular source of the huge increases in extracellular glutamate that occur during ischaemic cell death has previously been thought to be neuronal in origin but it has been suggested that from culture experiments that the microglia release massive amounts of the excitatory neurotransmitter and therefore may contribute to ischaemic injury (Wood, 1995). *In situ* hybridisation experiments for the pro-inflammatory cytokines suggest that they are produced primarily in the activated microglia and also show that the profile of expression is different following different insults i.e. motorneurone axotomy compared with traumatic spinal cord injury. This could perhaps suggests a different role in the different situations (Bartholdi & Schwab, 1997; Streit *et al.*, 1999). Following ischaemic insult the upregulation of the pro-inflammatory cytokines is usually of very short duration suggesting tight regulation of the response and providing support for the idea that the microglia may actually have a beneficial role (Buttini *et al.*, 1994; Woodroofe *et al.*, 1991).

Microglial activation in the brain is triggered by signals from injured neurones and may indeed be beneficial in certain scenarios i.e. where neurones are not lethally damaged (facial motor neurone lesions or in the ischaemic penumbra) they may play a role in tissue recovery and repair (Moore & Thanos, 1996; Raivich *et al.*, 1999). It has been proposed that transient activation is beneficial for brain repairing processes and chronic reactivity of the cells detrimental (Petrova *et al.*, 1999). *In vitro* findings that activated microglia produce neurotrophins such as nerve growth factor (NGF) and transforming growth factor β (TGF-β) suggest involvement in neuronal regeneration (Elkabes, 1996; Giulian *et al.*, 1993; Gebicke-Haerter *et al.*, 1996; Wahl *et al.*, 1991). The neuroprotective role of microglia is supported by the observation that in global cerebral ischaemia, neurones in the hippocampus

do not degenerate despite pronounced microglial activation (Abraham & Lazar, 2000). *In vitro* data clearly shows that in behaving as macrophages, microglia can secrete substances with proven neurotoxic actions suggesting that activation can be detrimental (Streit, 1993; Giulian *et al.*, 1993). Activated microglia in culture release reactive oxygen species, cytokines, glutamate, proteases, arachadonic acid derivatives and other small neurotoxic molecules (Paris *et al.*, 2000; Banati *et al.*, 1993; Colton & Gilbert, 1987; Wood, 1995; Giulian *et al.*, 1993; Gonzalez-Scarano & Baltuch, 1999). The extrapolation of *in vitro* data and interpretation of the results of various intervention studies in animal models that have been directed at molecules have lead to the idea that these molecules are produced by activated microglia *in vivo* (Lehrmann *et al.*, 1997).

The analysis of OX-42 stained sections clearly showed activation of the microglial cells following an ischaemic insult. OX-42 is generally a reliable marker of microglia and has previously been proved to be sensitive enough to show slight changes in microglial activation (Abraham & Lazar, 2000). Although taking sometime to establish, the initial experiments with the antibody showed clearly activated microglia in the ipsilateral cortex in the sections from animals that had been subjected to MCA occlusions. The administration of FK506 appeared to cause a de-activation or prevent activation of the cells in both the cortex and striatum. The 3 hr time point was initially chosen to examine the microglial response with a view to then examining any differences that might have occurred as a consequence of an hour reperfusion following a 2 hr transient occlusion. The idea that FK506 may mediate its differential neuroprotective effects demonstrated in the two models in *Chapter 4* through its action on the microglia. Unfortunately the staining of the cells as not consistent and did not allow such examination to be conducted. The problems with staining in the subsequent experiments were not due to the reagents used as these were changed and there was some staining of the sections although it was not consistent. The conclusions drawn from these experiments was that FK506 definitely caused an alteration in the activation state of the

microglia. Others, both *in vivo* and *in vitro*, have reported this observation. Herdegen *et al.* (2000) demonstrated microglial response to FK506 *in vitro* making the *in vivo* response of interest (Herdegan *et al.*, 2000). Following LPS stimulation *in vitro*, FK506 administration was able to prevent activation of microglial cells and addition cause re-ramification of activated cells following LPS exposure (Mielke *et al.*, 1999). FK506 administration *in vivo* has also been shown to reduce the microglial activation in the white matter of drug treated animals compared with vehicle treated controls (Wakita *et al.*, 1998; Herdegan *et al.*, 2000).

Further attempts to gain insight into the role of microglia in focal cerebral ischaemia using doxycycline were unfruitful. Doxycycline was included as it has previously been reported to completely prevent microglia activation and was neuroprotective in global brain ischaemia (Yrjanheikki *et al.*, 1998; Yrjanheikki *et al.*, 1999). The administration of the tetracycline compound in the monofilament MCA occlusions appeared to have no effect on the activation state of the microglia at either the 10 or 45 $mg.kg^{-1}$. This observation is in keeping with the results of the neuroprotection studies (*Chapter 4*) where doxycycline administration in both the permanent and transient monofilament MCA occlusions had no effect on the volume of ischaemic damage.

It was decided to simplify the experiments with the microglia and establish the activation state and effect of FK506 in culture with a view to interpreting the *in vivo* data. Using a culture system it has been possible to attempt to establish a relationship between morphology and function response (Nakajima & Kohsaka, 1998). In culture, isolated microglia assume the amoeboid morphology initially and with time they settle and take on more diverse morphologies. Difficulties in keeping isolated microglia in the resting state has been reported by other and it appears that the cells are extremely sensitive to the composition of the culture medium (Nakamura *et al.*, 1999). The conditions used to achieve ramification of the cells *in vitro* seem to vary between research groups with differences in media and flask types used

for culturing (Tanaka *et al.*, 1998; Tanaka *et al.*, 1999; Kim *et al.*, 2000; Petrova *et al.*, 1999). Some researchers have reported ramified microglia are best cultured in serum free medium on poly-L-lysine coated coverslips (Sudo *et al.*, 1998). Other authors suggest that non-coated glass and plastic substrates are strong activators of microglia and that coating with poly-L-lysine induces the ramified shape of microglia (Sudo *et al.*, 1998). In the experiments performed for this thesis, ramification was achieved in NUNC flasks using glial-conditioned medium having tried a number of different types of flask, different medium compositions and times between plating and experimentation. It may be difficult to interpret the morphological changes of cultured microglial as the cells may have already undergone activation through the somewhat brutal culturing procedure. They take on a typical macrophage-like phenotype and are therefore sometimes termed microglia derived brain macrophages (Streit, 1993; Streit *et al.*, 1999). Some authors report that microglia cultured on astrocytes monolayers become ramified and show morphology closer to that of resting microglia in the normal brain (Kloss *et al.*, 1997). In the normal brain, microglia are sometimes in contact with neurones and other cell types and it is suggested that the culturing process may alter the microglial response because of the altered physical relationship between cell types. Factors released from astrocytes and other cells in the brain may be absent in culture and so may alter the microglial response (Sudo *et al.*, 1998).

LPS is routinely used to investigate the activation of microglia *in vitro* (Nakamura *et al.*, 1999; Giulian & Vaca, 1993; Puffenbarger *et al.*, 2000; Sanz & Di Virgilio, 2000; Possel *et al.*, 2000; Zielasek & Hartung, 1996) and this was confirmed in the experiments conducted in this thesis. LPS has been shown to increase the production of cytokines, NO and the like when administered to cultured microglia (Loughlin & Woodroofe, 1996; Streit, 1993). Nakamura *et al.* (1999) reported changes in morphology of the cells in pure microglial cultures as early as 3 hrs after LPS stimulation (Nakamura *et al.*, 1999). Kim *et al.* (2000) injected LPS stereotaxically into the rat brain to investigate microglia reactivity and neuronal

susceptibility. They showed that there was region specific neuronal susceptibly to LPS induced neurotoxicity and suggested that this was related to the differences in microglial number and levels of inflammation related products produced by these cells (Kim *et al.*, 2000). The cultured microglia showed an altered morphology but this was difficult to assess quantitatively and was subjective. A more definitive answer would have been preferable. The staining with the OX-42 was unsuccessful despite reports by others of its use *in vitro*. OX-42 was previously been shown to intensely stain in microglia in primary cortical cultures (Booth & Thomas, 1991). Griess reagent, used to detect changes in NO (an indicator of activation) used to assess microglia in culture by others (Pyo *et al.*, 1999) and previously in this laboratory, provided no data in these experiments. It was thought that the lack of colour change that is the marker for production of NO was because the signal that was not sufficient to be detected. The number of cells was increased in the culture wells to increase the signal with little success. The administration of FK506 affected the morphology of the cells that were LPS stimulated although this again was a subjective assessment but was in keeping with the observations from Herdegen's group (Mielke *et al.*, 1999).

A series of experiments concerning the microglia and their contribution to the progression of ischaemic damage was performed with the technical help of Mrs Joyce McLuckie. With the increasing awareness of the use of animals in scientific experiments there have been attempts to simulate *in vivo* ischaemia experiments in the test tube i.e. *in vitro* (Home Office Recommendations). A number of experiments had previously been performed with the laboratory using an anoxic chamber to establish suitable protocols that mimicked the loss of substrates (glucose and oxygen) that occurs during an ischaemic insult and allowed assessment of neuronal viability with FITC-linked MAP-2 immunostaining. As stated before, *in vitro* systems have the advantage in terms of simplification and ease of manipulation but is an artificial environment and there observation made may not be truly representative of the *in vivo* state (Streit, 1993).

The previous oxygen-glucose deprivation (OGD) experiments showed that exposure to 0 % O_2 and 0.5 mM glucose produce neuronal cell death with 60 min and that the cells could be protected by MK-801 administration, which confirmed the excitotoxic nature of the cellular demise. FK506, which as stated before is a potent neuroprotectant, failed to protect the neurones from the insult (*G. May – unpublished data*). A further series of experiments proved that the addition of serum in the culture medium required longer OGD exposure times to produce neuronal death, proved MK-801 was still as effective at preventing neuronal cell death but still showed FK506 to have no effect. It was suggested that the effect of the serum on neuronal viability was related to its Ca^{2+} buffering capacity in the medium thereby reducing the severity of the insult (Kristian & Siesjo, 1998).

It has been established that the interaction of neurones and glial cells in culture can affect their response to stimuli (Tanaka *et al.*, 1999; Sudo *et al.*, 1998) and the previous studies on pure neuronal cultures, although providing some answers neglected the effect of the glial cells on neuronal survival. In order to investigate the effect of the glial cells, in particular the beneficial vs. detrimental role of microglia on neuronal survival, co-cultures of neurones and mixed natural glia and astrocytes and microglia alone were established and subjected to OGD. The hypoxia experiments that examined the effect of the glial cells *in vitro* suggested that the microglia had a beneficial effect, if only initially on neuronal viability. Although the neurones eventually all died by 90 min exposure to OGD it does suggest that the microglia were in some way supporting the neuronal cells. If this action of the microglia could be enhanced it could not only potentially have a beneficial effect on the outcome following insult but also suggests that the neurones that remain viable could also be targeted in an attempt to promote their survival. If the microglia are beneficial in the ischaemic brain it raises a question as to which other cells affected by the insult may be deleterious to the outcome.

CONCLUSIONS

The main conclusion from this chapter is that FK506 has an effect on microglial activation both *in vivo* and *in vitro*. *In vivo* data could suggest that microglial activation is deleterious and that deactivation by FK506 could potentially be the mechanism of observed neuroprotection. The *in vitro* ischaemic insult data suggested may that the microglia are beneficial immediately following an ischaemic insult. The time and effort spent in establishing the ramified microglial cultures was important for other work continuing in the laboratory investigating microglial activation. The activation state of the cell assessed by morphology was difficult to interpret conclusively but provide a lead for further investigation using a different approach.

CHAPTER 6

Cytokine analysis

INTRODUCTION

Cytokines are soluble factors, active at picomolar concentrations that can have diverse and frequently opposing effects on different cells. They are low molecular weight proteins that act as intracellular messengers in growth and differentiation and in immune responses in the CNS. They are generally produced in response to tissue injury and inflammation (Legos *et al.*, 2000; Rothwell *et al.*, 1996) and orchestrate immune responses of the body (Schobitz *et al.*, 1994; Sawada *et al.*, 1995; Kim, 1996; Arai *et al.*, 1990). The term cytokine was initially suggested in 1979 by Cohen *et al.* to define proteins that are derived for cells and influence the actions of other cells (Cohen *et al.*, 1979). This terminology united the ideas from as far back as the end of the 19th century that extended well into the 1960s, that molecules in the blood secreted by cells of the immune system affected other cell populations both in the immune system and outwith. The modern definition of the term cytokine is 'an inducible polypeptide/protein (glycoproteins) of molecular weight greater than 5 kDa that produces specific receptor mediated effects on target cells or on the producer cell' (Henderson & Poole, 1994). For a long time, the involvement of cytokines in brain injury was questioned (in keeping with the concept of immune privilege of CNS tissue (Becher *et al.*, 2000)) but it is now widely held that cytokines have diverse actions in the brain, modulating and mediating responses to disease and local changes related to inflammation, injury and infection (Rothwell, 1999).

The cytokines are classed pro- or anti-inflammatory depending on the sum total of their effects on immune cells (Vitkovic *et al.*, 2000). The pro-inflammatory cytokines include interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) (Clark, 1997) and are of particular interest in this thesis because of their involvement in the acute inflammatory response and their potential role in the development of ischaemic brain damage. In the periphery, the biological effects of these particular cytokines include wound healing, elimination of antigens, inflammation, vasodilation and haematopoiesis, whilst centrally they affect functions such as body temperature, feeding, nausea and sleep (Schobitz *et al.*, 1994). The specific role of an individual cytokine can shift from being beneficial to deleterious depending on the nature of the pathological state in which cytokines are involved (Turrin & Plata-Salaman, 2000; Van Wagoner & Benveniste, 1999).

The basal levels of expression of the cytokines in the normal brain are low but levels increase in response to injury or infection (Yu & Lau, 2000; Sharma & Kumar, 1998; Henderson & Poole, 1994; Rothwell, 1999). Cytokines both activate glial cells and cause the production and release of other cytokines in response to stimulation *in vitro* (Arvin *et al.*, 1996). Cytokines are involved in the development of the nervous system but have also been implicated in the pathology of autoimmune diseases of the CNS such as multiple sclerosis. Cytokines can enhance inflammation and exert direct toxic effects on cells and thereby promote the expansion of lesions playing a key role in the development of damage. Alternatively they may also limit the immune response and promote cell growth (Merrill & Benveniste, 1996). They are involved in the recruitment of leukocytes and increase the expression of adhesion molecules on leukocytes and endothelial cells (Arai *et al.*, 1990; Clark *et al.*, 2000).

The analysis of cytokine expression following focal cerebral ischaemia sometimes appear a confused issue as different authors suggest different time points at which the cytokines are first detected, peak and then subside. While these discrepancies may reflect the differences in different models and species used, they may be a consequence of the methods used to analyse the production and expression of the cytokines. However, the general consensus has led to speculation that the early induction of IL-1β, IL-6 and TNFα appears to initiate the inflammatory cascade following cerebral ischaemia (Kim, 1996).

THE ROLE OF CYTOKINES IN FOCAL CEREBRAL ISCHAEMIA

Focal cerebral ischaemia has been shown to elicit a notable cytokine response in the brain (Arvin *et al.*, 1996; Clark, 1997). The evidence for the involvement of cytokines in the pathology of focal ischaemia comes from clinical studies where elevated levels of the cytokines have been detected in the cerebrospinal fluid (CSF) and plasma of stroke patients which has been correlated with infarct size (Legos *et al.*, 2000). Evidence from pre-clinical studies supports the involvement of the cytokines in ischaemic damage and leads to the idea that anti-cytokine strategies may be of use in reducing lesion size.

INTERLEUKIN-1β

Interleukin-1 exists in two separate isoforms (α and β) which are the products of separate genes and play an important role in mediating the acute immune response (Hofman & Hinton, 1990). In the periphery, IL-1α and IL-1β are believed to exert identical actions (Dinarello, 1998). The IL-1α isoform remains membrane bound suggesting it has localised actions, whereas the β form is secreted (Schobitz *et al.*, 1994). Inactive or partially inactive precursors are produced by the cells and are then activated by cleavage by specific proteases.

Pro-IL-1α is biologically active whereas the IL-1β precursor is not and must be cleaved by IL-1β converting enzyme (ICE) (Rothwell, 1999). IL-1β is found as an inactive 31-33 kDa precursor, pro-IL-1β, which when cleaved produces a 17 kDa mature protein (The Cytokine Catalogue 2000). IL-1β expression appears to be associated with an increase in the expression of ICE and cleavage of the precursor (Bhat *et al.*, 1996). IL-1β receptor antagonist (IL-1ra) is an endogenous receptor antagonist that blocks the actions of the IL-1β and IL-1α in both the periphery and the brain (where it is mainly found in neurones) and functions as a very useful pharmacological tool to study IL-1 functions (Rothwell, 1999). A third member of the IL-1 family, interferon gamma inducing factor, also known as IL-18, was identified and cloned from mouse liver. Due to its close homology with the others members of the family, it was termed IL-1γ (Bazan *et al.*, 1996). In contrast to the other two isoforms, IL-1γ/IL-18 is constitutively expressed in the brain (Culhane *et al.*, 1998) although the exact function of this cytokine in CNS physiology and pathophysiology has not been fully determined (Touzani *et al.*, 1999).

The β isoform of IL-1 is constitutively expressed at a low level in the normal brain and is the predominate form expressed upon pro-inflammatory stimulation (Eriksson *et al.*, 2000; Touzani *et al.*, 1999). IL-1β is thought to be produced by various elements of the CNS including neurones, microglia, astrocytes and the endothelium (Rothwell, 1991). In addition, mice deficient in the ICE gene have smaller lesions and are not able to process IL-1β and have altered expression of ICAM-1 suggesting these two proteins are related following ischaemia (Yang *et al.*, 1999).

At the pharmacological level, IL-1 acts via two main receptors. The type I receptor is present in many cells and binds the two isoforms of IL-1 with similar affinity. The type II receptor is found on the surface of immune cells such as neutrophils and macrophages and has greater

affinity for IL-1β (Dinarello, 1991). The intracellular signal transduction cascade following the IL-1β binding to its receptors in immune cells includes a cyclic AMP (cAMP) and protein kinase C response followed by protein phosphorylation (Dinarello, 1991). This is then followed by the induction of immediate early genes such as c-*jun* and c-*fos* (Vitkovic *et al.*, 2000).

Induction of IL-1β has been demonstrated following both permanent (Buttini *et al.*, 1994; Liu *et al.*, 1993) and transient MCA occlusion (Wang *et al.*, 1994a) although attempts to detect IL-1β in the blood of stroke patients have not be successful, presumably because the levels are low in the plasma (Fassbender *et al.*, 1994). In experimental ischaemia, IL-1β mRNA expression occurs as early as 15 min post-occlusion, peaks rapidly (3-6 hrs) and return to basal levels at 12 hrs in the ischaemic zone. Expression appeared to be more pronounced in the transient model (Buttini *et al.*, 1994; Wang *et al.*, 1994a). In a study using a multiprobe RNAse protection assay, Hill *et al.* (1999) showed the temporal expression of IL-1β, TNFα and IL-6 in mouse models of focal ischaemia. They demonstrated that TNFα and IL-6 were elevated early in both the transient and the permanent model. IL-1β showed a temporal shift that was model dependent where reperfusion in the transient model was associated with the delayed expression of the cytokine (Hill *et al.*, 1999) (***Figure 40***). IL-1β protein has been shown, by bioactivity assay, to be maximal 6-8 hours in the ischaemic hemisphere following an insult (Rothwell, 1999). Davies *et al.* (1999) demonstrated by immunocytochemistry that IL-1β protein was localised to microglia/macrophages 1 hr following permanent MCA occlusion in the rat, with delayed expression associated with invading immune cells and astrocytes (Davies *et al.*, 1999).

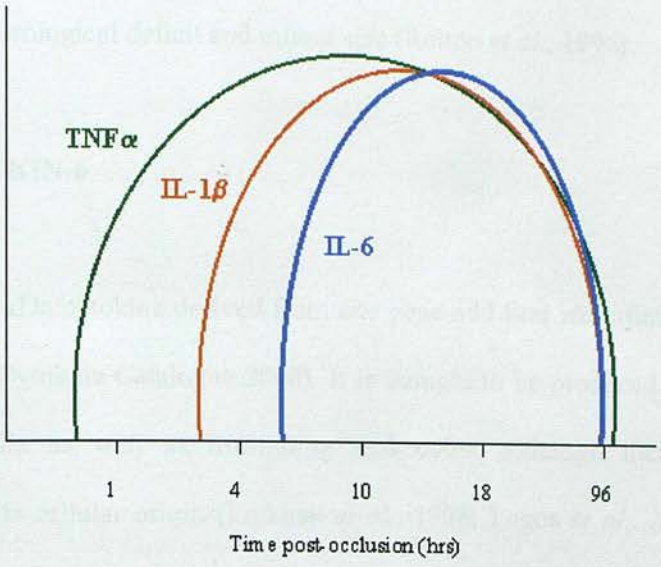


Figure 40. Schematic representation of mRNA expression in the mouse following transient focal cerebral ischaemia. Theoretical model of gene expression of the temporal relationship of TNFα, IL-1β and IL-6. In the permanent model the mRNA of the three cytokines is detected slightly later (Hill *et al.*, 1999).

Experiments *in vitro* to elucidate the mechanism of IL-1β actions in cell death have provided conflicting results. In primary neuronal cultures, IL-1ra does not inhibit cell death induced by NMDA or AMPA receptor agonists nor does IL-1β enhance it (Rothwell, 1999). Exogenous IL-1β proved to be neuroprotective in the nM range whereas higher concentrations (mM) proved neurotoxic (Rothwell & Strijbos, 1995). In mixed cultures, IL-1β is toxic at picomolar concentrations, which is thought to be due to the release of secondary mediators such as NO and free radicals (Benveniste, 1992; Chao *et al.*, 1995; Chao *et al.*, 1996). IL-1β is however not toxic to normal adult brain *in vivo* (Rothwell, 1999) and as always *in vitro* data should be interpreted with caution. The administration of recombinant IL-1β prior to MCA occlusion caused increase damage, BBB breakdown, leukocyte infiltration and oedema formation whereas antibodies against IL-1β appeared to

reduce damage (Yamasaki *et al.*, 1995). Experimental treatment with IL-1ra has been shown to reduce neurological deficit and infarct size (Relton *et al.*, 1996).

INTERLEUKIN-6

IL-6 is a 26 kDa cytokine derived from one gene and first identified as a B cell stimulating factor (The Cytokine Catalogue 2000). It is thought to be produced by neurones, astrocytes and microglia as well as infiltrating leukocytes, although there is much discussion concerning its cellular origin (Loddick *et al.*, 1998; Legos *et al.*, 2000). Its wide range of biological actions is similar to those of IL-1β and like IL-1β expression also occurs in different models of brain injury. However, in contrast to IL-1β and TNFα, it has been suggested that IL-6 has both pro- and anti-inflammatory properties (Arvin *et al.*, 1996) with both neuroprotective and neurotrophic actions (Block *et al.*, 2000). IL-6 initiates biological activity by binding to a high affinity complex of two membrane glycoproteins, low affinity 80 kDa membrane bound receptor (IL-6R) and a high affinity 130 kDa component (gp130) (Clark, 1997). In stroke patients, increased levels of IL-6 have been detected in the plasma and CSF and were associated with larger infarcts (Tarkowski *et al.*, 1995).

In both mixed and pure neuronal cultures, IL-6 dose dependently protected the cultures from the NMDA toxicity that was blocked by a competitive IL-6 inhibitor but did not protect against serum withdrawal and staurosporine induced apoptotic cell death. These results suggest that IL-6 may be involved in endogenous neuroprotection in NMDA mediated injury (Ali *et al.*, 2000). It has been suggested that IL-6 may mediate neuroprotection by inhibition of IL-1β production (Schindler *et al.*, 1990) or through the induction of IL-1ra (Tilg *et al.*, 1994). Cortical neuronal cultures pre-treated with IL-6 were resistant to excitatory amino acid induced toxicity (Rothwell & Strijbos, 1995). Higher concentrations of the cytokine

have however also been shown to be toxic to cultured cells and mice that overexpress IL-6 show early neurodegenerative changes (Clark *et al.*, 2000). In studies of neuronal regeneration following axotomy in rat facial neurones, IL-6 was shown to be upregulated which was associated with a microglial reaction. Regeneration supporting the idea that IL-6 is beneficial and neuron derived IL-6 causes microglia to proliferate (Streit *et al.*, 2000).

TUMOUR NECROSIS FACTOR α

In the periphery, TNFα is involved in septic shock, haematopoiesis and has anti-tumour effects and a diverse array of biological activities including acute phase protein secretion, vascular permeability and activation of inflammatory cells (Tracey & Cerami, 1993). Produced predominately by activated monocytes/macrophages, human TNFα is synthesised as a 25 kDa pro-cytokine, which is processed to yield an active 17 kDa product (Hofman & Hinton, 1990). During ischaemia, TNFα is synthesised as a precursor peptide pro-TNFα, which is membrane bound and then cleaved by proteolytic enzymes (Shohami *et al.*, 1999). TNFα effects are mediated via specific receptors of which the death receptors (p75 and p55 proteins) are the best known (Smith *et al.*, 1994). Binding of TNFα to its receptors stimulates activation of a variety of kinases via the recruitment of a number of adapter proteins and the activates of several transcription factors including NF-κB known to be involved in the expression of both adhesions molecules and other cytokines (Heller & Kronke, 1994).

TNFα has been shown to be elevated in several models of brain injury including LPS administration, trauma and administration of neurotoxins by RT-PCR, by western blotting and by bioactivity assay (Shohami *et al.*, 1999; Shohami *et al.*, 1994; Arvin *et al.*, 1996). TNFα plasma levels have been found to be higher in acute stroke patients than control

patients supporting its role in the pathology of the disease (Elneihoum *et al.*, 1996). During the early phase of indirect brain injury such as that occuring following the facial axotomy, TNFα is absent which may suggest that its function is restricted to severe forms of brain pathology (Raivich *et al.*, 1999). In a permanent model of cerebral ischaemia, elevated levels of TNFα mRNA have been detected in the infarcted zone as early as 1 hr post-occlusion (prior to influx of PMNLs), peaking at 12 hrs and persisting for 5 days (similar expression to IL-1β) with no expression in the contralateral hemisphere (Liu *et al.*, 1994). The cellular source of TNFα is also a debated topic. Liu *et al.* (1995) propose that neurones initially produce the cytokine followed by infiltrating inflammatory cells whereas others suggest neurones, astrocytes and microglia (Botchkina *et al.*, 1997; Liu *et al.*, 1994; Tseng & Chang, 1999). Uno *et al.* (1997) reported that TNFα was expressed in both microglia and astrocytes but the spatial and temporal expression was different perhaps indicating different functions in the release of the cytokines by these two cells types (Uno *et al.*, 1997). Buttini *et al.* (1996) identified the upregulation of TNFα mRNA and protein in activated microglia and macrophages (Buttini *et al.*, 1996) suggesting that TNFα is part of the endogenous inflammatory response. In this respect, TNFα may play a primary role in setting the stage for inflammatory and immune reactions elicited by cerebral ischaemia (Feuerstein *et al.*, 1994). TNFα may prime the brain for subsequent damage by activating capillary endothelium to a pro-adhesive state (del Zoppo *et al.*, 2000) by upregulating the expression of cell adhesion molecules (Arvin *et al.*, 1996). Shohami *et al.* (1999) suggested that TNFα knockout animals showed improvement in function and smaller lesion size initially supporting the toxic nature of TNFα acutely but pointed out that the animals greater neurological dysfunction at a later point implying that the cytokine may be required for repair mechanisms following the insult (Shohami *et al.*, 1999).

Studies where TNFα was administered prior to the occlusion of the vessel in ischaemia showed exacerbation of damage (Arvin *et al.*, 1996). TNFα antibodies have been shown to attenuate tissue damage which strengthens the case for the involvement of the cytokine in the development of the lesion (Barone *et al.*, 1997; Lavine *et al.*, 1998; Pettigrew *et al.*, 1999). Conversely, evidence from a study in mice showed that when TNFα was pre-administered it appeared to have a protective effect against the ischaemic insult (Nawashiro *et al.*, 1997). Further evidence for a role of TNFα in ischaemia is supported by findings from studies with transgenic animals. Mice lacking both the p77 and p55 TNFα receptors develop greater infarcts supporting the protective role of the cytokine (Bruce *et al.*, 1996). Interestingly, the microglial immunoreactivity in the TNFα knockout animals was reduced, perhaps implicating the cytokine in microglial activation. It has been suggested that TNFα, in a beneficial role, may act synergistically with IL-1β to produce NGF and exert neuroprotective actions (Gadient *et al.*, 1990) and can induce the production of IL-6 (Hofman & Hinton, 1990). Others, utilising knockout technology, propose that TNFα expression is not related to or upregulated by IL-1β (Yang *et al.*, 1999). Like IL-1β, TNFα could act as a cytotoxic agent or a growth factor as in culture it can cause cells to proliferate or die (Hofman & Hinton, 1990). Recently, Hu *et al.* (2000) showed IL-1β and TNFα together resulted in marked neuronal injury through the production of NO (Hu *et al.*, 2000). It has been suggested that the appropriate setting of mediators following brain injury determines whether TNFα is protective or toxic (Shohami *et al.*, 1999).

MOLECULAR ANALYSIS OF CYTOKINES

In summary, the data concerning cytokine expression during and following an ischaemic insult is, at best confusing, both in terms of the source and in terms of action. In the previous chapter where the microglial response to an ischaemic insult was examined histologically,

both *in vivo* and *in vitro,* the results were inconclusive. Another approach was sought in order to analyse the microglial response. The aim of these experiments was to establish the protocol for culturing microglia and assessing their morphology. The response of the cells to an LPS challenge was then examined to serve as a benchmark for the cytokine response. The aim was then to extend these studies to examine the effect of LPS stimulation *in vivo* and from there into the *in vivo* stroke models. In both the *in vitro* experiments and the MCA occlusion experiments, the effect of FK506 on the production of cytokines was also of interest in an attempt to extend the idea of the drug acting by inhibition of an inflammatory response.

MATERIALS & METHODS

CYTOKINE mRNA ANALYSIS

TISSUE PREPARATION FOR RT-PCR

Brain samples from LPS/saline treated animals

LPS or saline was administered to experimental animals as detailed in *Chapter 2*. Animals were sacrificed by decapitation and in each case the brain was rapidly removed, placed in a brain block and sectioned. The 2 mm section at the level of the MCA was selected and the right (ipsilateral in occlusion animals) cortex and striatum and corresponding left (contralateral in occlusion animals) regions were dissected out and snap frozen. Samples were stored at -70°C until required. Tissue was thawed on ice in 350 µl of Buffer RLT (Qiagen). Samples were homogenised using a rotor stator homogeniser (Powergen 35, Fisher Scientific) and the lysates stored at -70°C. When required, the frozen lysates were thawed,

incubated at 37°C for 10 min and centrifuged at maximum speed for 3 min (eppendorf Centrifuge 5415C). The supernatants were retained and used in the subsequent steps.

Brain samples from MCA occlusion animals

Brain samples were obtained as described for LPS and saline treated animals detailed above.

Microglial cell culture samples

Culture flasks were scraped with a cell scraper (Costar 3010) to remove attached microglial cells. The medium was collected and centrifuged at 500 x g for 10 min (Mistral 2000, MSE). Supernatants were removed the pellet from centrifugation of the culture medium containing microglial cells was disrupted by the addition of the appropriate volume (350 µl) of Buffer RLT. Lysates were homogenised using QIAshredder columns (Qiagen) centrifuged at maximum speed.

EXTRACTION OF RNA

The manufacturer's protocol for the isolation of total RNA using RNeasy reagents (Qiagen) was followed. An equal volume of 70 % ethanol was added to the brain tissue supernatants and the homogenised microglial lysates and mixed well using a pipette. 700 µl of each sample was added to an RNeasy mini spin column and centrifuged at 8000 x g for 15 s. Each column was washed by addition of 700 µl of Buffer RW1 followed by centrifugation at 8000 x g for 15 s. The RNeasy columns were transferred to new 2 ml collection tubes and 500 ml of Buffer RPE added and the columns centrifuged as before. 500 µl of Buffer RPE was added to the columns that were centrifuged at maximum speed for 2 min to dry the

membranes. Columns were transferred to a 1.5 ml collection tubes and 30 µl of RNase-free water added directly to the membranes and centrifuged at 8000 x g for 1 min to elute the RNA. An aliquot of each sample was diluted 1:50 in RNase free water and used to quantify and determine purity the total RNA by spectrophotometry (Double Beam Spectrophotometer CE 594, Cecil Instruments). One absorbance unit at 260 nm (A_{260}) corresponds to 40 $\mu g.ml^{-1}$ of total RNA and the ratio of the absorbance at 260 nm and 280 nm (A_{280}) gives an estimate of RNA purity. The remaining samples were stored at -70°C.

FORMALDEHYDE AGAROSE GEL ELECTROPHORESIS

The quality and intactness of the extracted RNA was checked on an agarose gel. Formaldehyde agarose gels (*Appendix 3*) were poured, placed in the electrophoresis chamber (GibCoBRL Horizon 58, Life Technologies), covered by 1 x RNA running buffer *(Appendix 3)* and the samples loaded. Gels were run at 1-5 V/cm (typically 100 V) for approximately 1 hr. The gel was photographed using a 3UV™ Transilluminator and Polaroid Camera with Polaroid Black and White film type 667 (Sigma).

REVERSE TRANSCRIPTION

Reverse transcription to synthesise a template (copy DNA, cDNA) for the amplification of the genes of interest by polymerase chain reaction (PCR) was performed using First-Strand cDNA Synthesis reagents (Amersham Pharmacia Biotech). Total RNA from the test sample (1-5 µg) was placed in a 0.5 ml eppendorf centrifuge tube and RNase-free water added to bring the volume to 8 µl. The mixture was heated to 65°C for 10 min and then chilled on ice. The heat denatured RNA was added to 5 µl of Bulk First-Strand cDNA Reaction Mix, 1 µl of DTT solution and 1 µl of *Not* I-$d(T)_{18}$ at a final concentration of 0.2

$\mu g.ml^{-1}$, pipetted gently to mix and incubated at 37°C for 1 hr. Samples were stored at 4°C until required for PCR.

OLIGONUCLEOTIDE PRIMERS

The experiments to analyse inflammatory cytokine expression from both microglial cultures and rat brain tissue required oligonucleotide primers for 3 cytokines of interest to be designed. The primers for IL-1β, IL-6 and TNFα were based on the sequences in a paper by Delgado-Rizo *et al.* (1998) (*Appendix 3*) (Delgado-Rizo *et al.*, 1998). The primers were designed to contain a high GC component because of the stability this lends to the annealing steps of the PCR process as a consequence of the triple bonds formed they form. The sequences were also chosen to limit primer dimer formation. The primers were synthesised by Life Technologies. The primers for the β-actin routinely used in the laboratory were purchased from Promega and were based sequences from Yamamura *et al.* (1991) (Yamamura *et al.*, 1991).

Below are detailed the sequences for the sense and antisense primers for each cytokine, the size of the expected fragment in base pairs (bp), the Genbank accession number and the melting temperatures (Tm) each pair.

Interleukin-1β Genbank Acc. #: E01884

(522 bp) Tm 61.9°C, 59.8°C

Sense 5' CCA GGA TGA GGA CCC AAG CA 3'
Antisense 3' CC TTT GTC GTT ACC AGC CCT 5'

Interleukin-6 Genbank Acc. #: M26744

(496 bp) Tm 59.8°C. 59.8°C

Sense 5' CTT CCA GCC AGT TGC CTT CT 3'
Antisense 3' GG GGT TGA AGG TTA CGA GAG 5'

Tumour Necrosis Factor α Genbank Acc. #: X66539 / S40199

(468 bp) Tm 64°C, 64°C

Sense 5' CGA GTG ACA AGC CCG TAG CC 3'
Antisense 3' CC GTC GAC CGC ACA AGT AGG 5'

β-Actin (285 bp) Tm 62°C, 65.1°C

Sense 5' TCA TGA AGT GTG ACG TTG ACA TCC GT 3'
Antisense 3' CCT AGA AGC ATT TGC GGT GCA CGA TG 5'

POLYMERASE CHAIN REACTION

RT samples were heated at 90°C for 5 min to denature the cDNA and inactivate the reverse transcriptase. A master mix (*Appendix 3*) was prepared for each cytokine and added to the 2-3 µl of the template to give 50 µl of reaction mix. In each experiment, negatives control (no template), positive controls where available and a house-keeping gene (β-actin) were included. Reaction mixtures were placed in Hybaid Touchdown PCR machine. Amplification conditions were as follows: 94°C for 5 min (denaturation), 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min (denaturation, annealing and polymerisation) and finally 72°C for 7 min.

AGAROSE GEL ELECTROPHORESIS

Agarose gels (*Appendix 3*) were poured, submerged in the electrophoresis chamber (GibCoBRL Horizon 11-14, Life Technologies) in sufficient 1 x Tris-[hydroxymethyl]-aminomethane (Tris) acetate EDTA buffer (TAE, *Appendix 3*) and the samples loaded. The gel was run at 1-5 V/cm typically 100 V for approximately 1 hr. The gel was photographed using a 3UV™ Transilluminator and Polaroid Camera with Polaroid Black and White film type 667 (Sigma).

MULTIPLEX PCR ANALYSIS

The initial experiments with the primers that had been designed in house looked promising but the availability of new reagents, the experiments were altered and a multiplex PCR (MPCR) system used. This allowed analysis of a number of genes in a single reaction thereby increasing output.

Total RNA was extracted, purified and quantified as described before using RNeasy reagents (Qiagen). MPCR reactions were set up with the test samples and the positive control (supplied), MPCR buffers (*Appendix 3*) and the appropriate primer mixtures from the Multiplex PCR Kit for Rat Inflammatory Genes Set 1 (rINF1G-MPCR Cat #: INF-M210G, Maxim Biotech, Inc., USA).

rINF1G-P001 10x rINF1G MPCR Rat Primer Mixture

	PCR Product Size	Genbank Acc. #	Tm (°C)
rINF1G-GMC (GM-CSF)	210 bp	U00620	68.5, 71
rINF1G-TGF (TGF-β)	250 bp	X52498	66.3, 69
rINF1G- IL1 (IL-1β)	294 bp	M98820	66, 69
rINF1G-TNF (TNFα)	351 bp	X66539	70.4, 69.8
rINF1G-IL6 (IL-6)	453 bp	M26744	66.4, 68.2
rINF1G-GAP (GAPDH)	532 bp	M17701	64, 65

A 'hot start' PCR protocol was used. Amplification conditions were as follows: 2 cycles of 96°C for 1 min, 59°C for 4 min followed by 30 cycles of 94°C for 1 min, 59°C for 2.5 min, 70°C for 10 min and finally a 20°C soak. The products of the MPCR were separated by electrophoresis on a 2 % agarose gel and the photographed as previously.

'QUANTIFICATION' OF MPCR ELECTROPHORESIS

A system was devised to interpret the results of the MPCR electrophoresis to allow graphical analysis of the gels. Ideally analysis of gene products run on electrophoretic gels should be quantified with equipment such as a phosphoimager. At the time of these experiments such technology was unavailable in the laboratory. Each gel photograph was analysed by eye by two independent observers (Jennifer McCarter and Lorraine Kerr). The expression of each gene was independently assessed and assigned a number between 1 and 3 depending on the intensity of the band relative to the other bands. Results from the two observers were then compared, all bands were then normalised against the GAPDH signal and plotted using

SigmaPlot. The use of GAPDH as a house-keeping gene has been demonstrated previously and is a valid marker against which expression can be normalised (Chapman *et al.*, 2000).

CYTOKINE WESTERN ANALYSIS

TISSUE PREPARATION FOR WESTERN ANALYSIS

Brain samples from MCA occlusion animals

Tissues from MCA occlusion operated and sham animals for western analysis was obtained in the same manner as described in Tissue Preparation for RT-PCR. For homogenisation, samples were thawed on ice, homogenised with a hand-held homogeniser (Kontes) in 50 µl of extraction buffer (EB) (*Appendix 4*). 2 µl of homogenate was withdrawn for a protein assay and the remaining homogenate stored at -70°C.

Microglial cell culture samples

Culture flasks were scraped with a cell scraper (Costar 3010) to remove attached microglial cells. The medium was collected and centrifuged at 500 x g for 10 min (Mistral 2000, MSE). Supernatants were removed and retained and the pellet homogenised with a hand-held homogeniser in 30 µl of EB. Again 2 µl of homogenate was withdrawn for a protein assay and the remaining homogenate stored at -70°C.

PROTEIN ASSAY

2 µl of each homogenate was diluted 1:50 in EB containing no inhibitors or PMSF. Colourimetric bicinchoninic acid (BCA) protein reagent (Pierce and Warriner UK Ltd) was used to determine protein concentration. 10 µl of supernatant was incubated with 200 µl of BCA reagent (mixed 1:50 as per instructions) at 37°C for 30 min. All samples were assayed in duplicate and EB was used as a blank. BSA protein standards (supplied with BCA reagent) were included on the microtitre plate (0.03-1 $mg.ml^{-1}$). Colour changes were measured at 560 nM in a Dynatech MRX machine and Revelation software.

WESTERN BLOTTING

For each immunoblotting experiment, a 12 % acrylamide gel with a 4 % acrylamide stacking gel was placed in Hoefer Mighty Small SE250/SE260 mini-gel apparatus with running buffer (*Appendix 5*). Samples for analysis, previously diluted in sterile PBS and aliquotted to give 60 µg of protein per lane and stored at -70°C, were defrosted on ice. Rat recombinant cytokine standards, IL-1β, IL-6 and TNFα (R&D Systems), were diluted from the relevant stock solutions to give 10, 20 and 25 ng respectively (appropriate protein concentration to allow detection). The volume of the samples and standards was doubled with sample buffer (*Appendix 5*) and all were heated to 95°C in Hybaid PCR machine for 20 min, subjected to a pulse spin and loaded onto the gel. Full range colour molecular markers (Amersham RPN 756) were loaded.

The gels were routinely run at 70 V initially and then at 150 V (Hoefer Mighty Slim SX250) for 2-3 hrs. The gels were removed, rinsed gently in transfer buffer (*Appendix 5*) and placed on prepared Millipore Immobilon-P polyvinylidene fluoride membrane (PDVF, 0.45µm)

between filter paper soaked in transfer buffer. Each gel sandwich was placed in the semi-dry transfer apparatus (Sigma-Aldrich) which was run at 15 V for 1 hr. Membranes were removed from transfer apparatus and placed in 5 % milk powder (BioRad) in Tris buffered saline-Tween 20 buffer (TBST, *Appendix 5*) for 1 hr at room temperature on a rotating platform to block non-specific sites. Initially, blotted gels were stained with Coomassie Bright Blue (Sigma) or silver stained (*Appendix 5*) to confirm effective protein transfer to the membrane.

Following blocking, membranes were incubated with appropriate anti-rat anti-cytokine antibodies (R&D Systems) diluted 1:500 (final concentration of 0.4 $mg.ml^{-1}$) in TBST/milk powder overnight at 4°C on rotating platform. Membranes were washed twice (2 x 10 min) in TBST and then incubated with an anti-goat/sheep IgG peroxidase conjugate monoclonal secondary antibody (Sigma, 1:10 000 in TBST/milk powder) for 2 hr at room temperature on rotating platform. Membranes were washed twice (2 x 10 min) in TBST and then in TBS pH 8. Protein bands on the membranes were visualised LumiGLO (New England BioLabs Inc.) and Hyperfilm ECL™ (Amersham 3114H).

CYTOKINE ENZYME-LINKED IMMUNOSORBENT ASSAY

Brain tissue samples were collected as described previously (*see Tissue Preparation for RT-PCR/Western Analysis*). Each sample was homogenised with a hand-held homogeniser in 100 µl extraction buffer containing no inhibitors (*Appendix 4*) and then centrifuged briefly (Micro Centaur, MSE). Supernatants from each sample were collected and assayed to establish protein concentration (*see Protein Assay*).

Cytokine ELISA assays were performed using Quantikine® M Murine Rat IL-1β (RTA00) and TNFα (RLB00) Immunoassay reagents (R & D Systems) according to the manufacturer's protocol (*Appendix 6*). Briefly, 50 µl of appropriate Assay Diluent was added to each well of a microplate pre-coated with cytokine monoclonal antibody for relevant cytokine followed by 50 µl cytokine standard or sample (150 µg of protein added per well) in duplicate, covered with an adhesive strip and incubated for 2 hrs at room temperature. Each well was aspirated and washed 5 times with 350 µl of Wash Buffer. Rat antibody conjugate (100 µl) for either IL-1β or TNFα was added to the wells in the microplate that was covered and incubated at room temperature for 2 hrs. The aspiration/wash step was repeated followed by the addition of 100 ml of Substrate Solution and 30 min incubation at room temperature. The reaction was halted by the addition of 100 µl of Stop Solution and the optical density in each well determined using the microplate reader at 450 nm (Dynatech MRX machine).

RESULTS

CYTOKINE mRNA ANALYSIS

Initial experiments using the primers that had been designed and synthesised were promising (***Figure 41***). Cortical tissue from the LPS treated animals showed gene fragment products corresponding to the sizes expected (~522 bp for IL-1β; ~496 bp for IL-6 and 285 bp for β-actin). However, no clear signal was seen for TNFα suggesting that either the amplification conditions were not optimal and would need refining or that there was no TNFα response. For each cytokine, a non-reverse transcription sample and control water sample was included confirming no contamination of the samples during the assay. The presence of the β-actin fragment as a house-keeping gene also confirmed successful reverse transcription from the mRNA.

In 1999, multiplex PCR reagents for the cytokines of interest became commercially available. A principle advantage of multiplex assays is that they permit the analysis of a number of gene products from a single sample and reduce tissue required. Initial results with the MPCR reagents using LPS/saline treated rat brain tissue confirmed the results from the earlier experiments with the primers designed in-house (***Figure 42***). In the LPS stimulated tissue, IL-6, TNFα and IL-1β at 3 hrs post stimulation. The detection of a TNFα response using the MPCR reagents supports the view that the amplification conditions in the initial experiments with synthesised primers were not optimal. The effect of LPS on the cytokine expression was evident in both cortical and striatal tissue. The expression of TGF-β was also elevated in all regions of the brain following LPS stimulation (***Figure 43***).

The effects of MCA occlusion on cytokine mRNA levels in the cortex (***Figure 44***) and striatum (***Figure 46***) was then performed using MPCR reagents. In the transient monofilament, TNFα expression in the cortex was detected at 3 but not at 24 hrs. There was IL-1β expression in the transient model at 3 and 24 hrs. There was no expression of IL-1β, IL-6 or TNFα in the cortex in the permanent model at 3 hrs but both IL-1β and TNFα were present at 24 hrs (***Figure 45***). Interestingly, whilst IL-6 was detected in LPS treated animals, it was not detected in either of the MCA occlusion models at either time point. The expression of the inflammatory cytokines in the striatum of transient monofilament occlusion at 3 hrs was no different from that seen in the sham group. At 24 hrs the expression of IL-6 and IL-1β had decreased, as had TGF-β. Similarly in the striatum of both the 3 and 24 hr permanent MCA occlusion groups, there was no expression of IL-1β, TNFα or IL-6 and TGF-β expression (***Figure 47***).

MPCR experiments conducted with the microglial cultures showed an increase in IL-6 expression following LPS stimulation at both 3 and 24 hrs (***Figure 48***). IL-1β, TNFα and TGF-β showed no change in expression levels between the two treatments at 3 hrs although there appeared to be an increase at 24 hrs in IL-1β and TNFα. Granulocyte macrophage colony stimulating factor (GM-CSF) expression was increased by LPS administration at both 3 and 24 hr. The administration of FK506 and its vehicle had little effect on the expression of any of the gene analysed at 3 hrs. At 24 hrs post LPS stimulation, FK506 administration appeared to reduce IL-6 expression only (***Figure 49***).

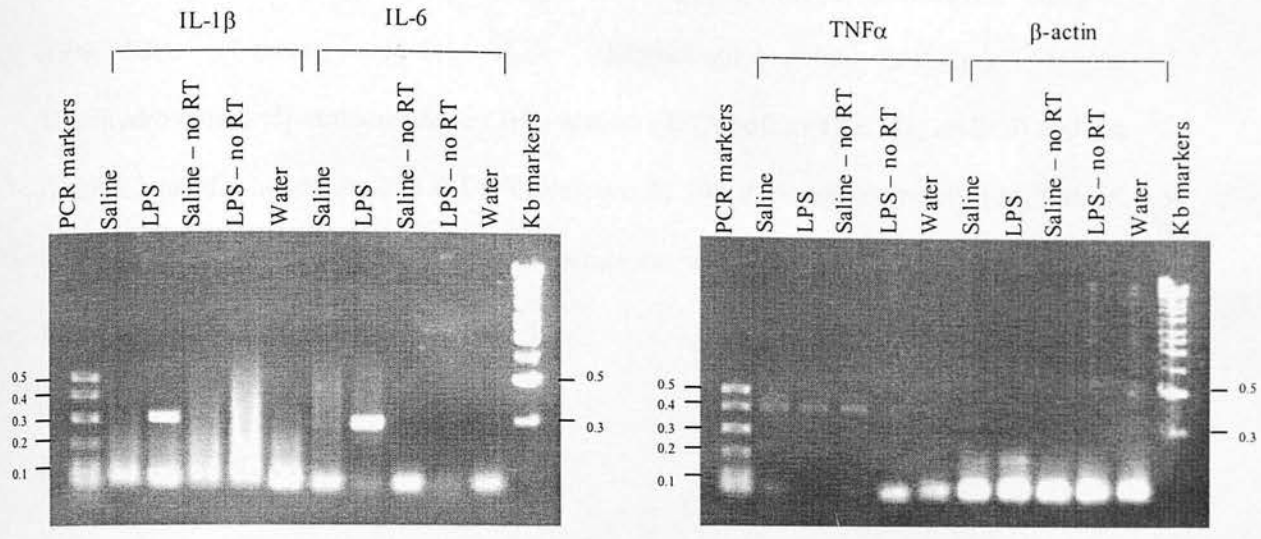


Figure 41. Agarose gel photographs showing PCR fragments for IL-1β and IL-6 (a) and TNFα and β-actin (b) in rat cortical tissue following saline or LPS stimulation. For each cytokine, a non-reverse transcription sample for each treatment group (lanes 4, 5, 9, 10, 16,17, 21, 22) and a control water sample (lanes 11, 23) were included. Positive signals are visible in the LPS stimulated tissue for IL-1β (522 bp; lane 3) and IL-6 (496 bp; lane 8). No clear signals were detected in the TNFα sample (lane 15) suggesting that the amplification conditions were not optimal. A 285 bp fragment for the house-keeping gene β-actin (lanes 19, 20) confirmed successful reverse transcription from the mRNA.

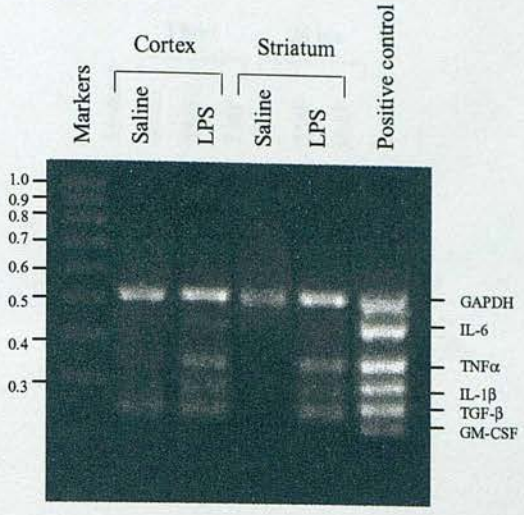


Figure 42. Agarose gel photograph showing the results of multiplex PCR for the inflammatory cytokines in tissue from an LPS and saline treated rat.

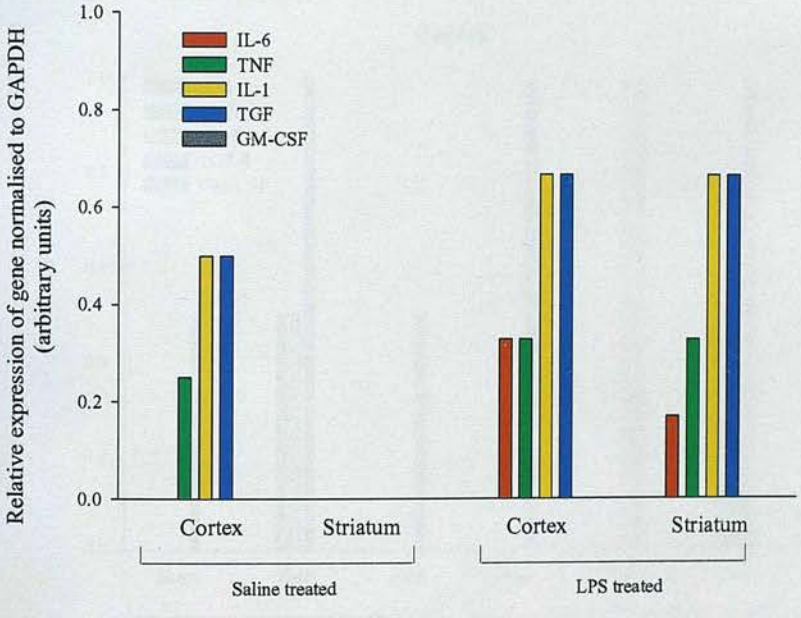


Figure 43. Graphical analysis of MPCR electrophoresis gel showing expression of inflammatory cytokines in rat brain following 3 hrs LPS stimulation.

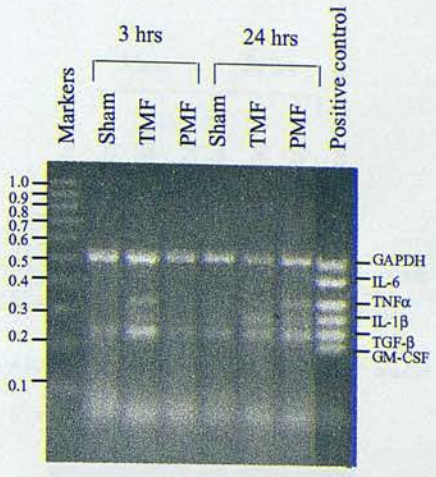


Figure 44. Agarose gel photograph showing the results of multiplex PCR for the inflammatory cytokines in the cortex of animals subjected to sham, transient (TMF) and permanent (PMF) monofilament MCA occlusion (3 and 24 hrs).

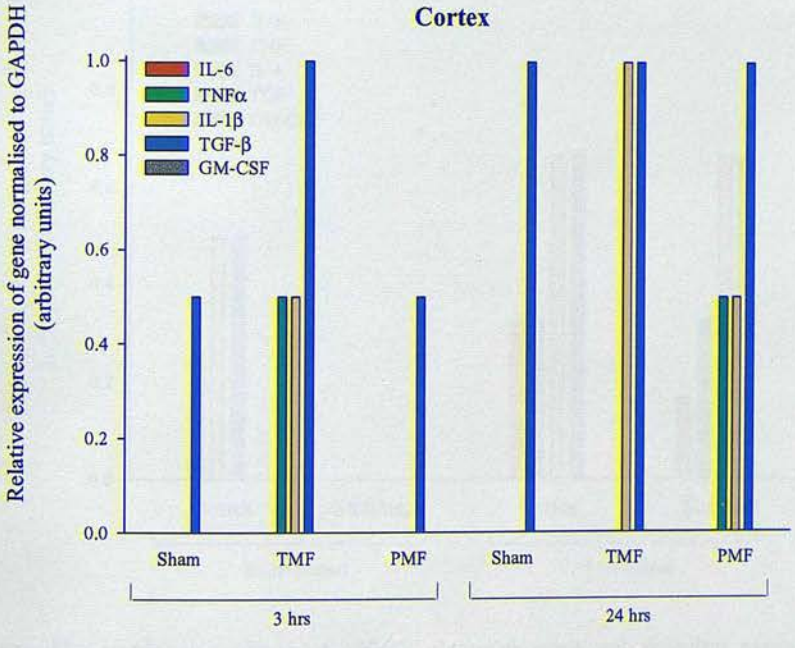


Figure 45. Graphical analysis of MPCR electrophoresis gel showing expression of inflammatory cytokines in the cortex following sham, transient (TMF) and permanent (PMF) MCA occlusion at 3 and 24 hrs.

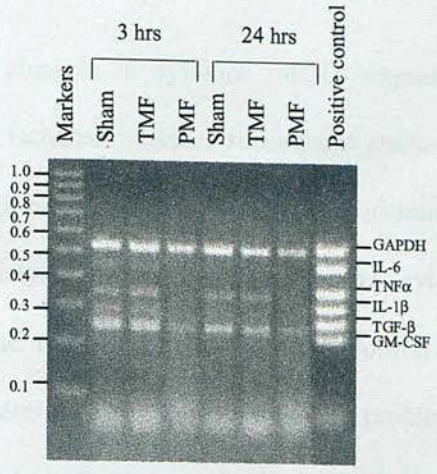


Figure 46. Agarose gel photograph showing the results of multiplex PCR for the inflammatory cytokines in the striatum of animals subjected to sham, transient (TMF) and permanent (PMF) monofilament MCA occlusion (3 and 24 hrs).

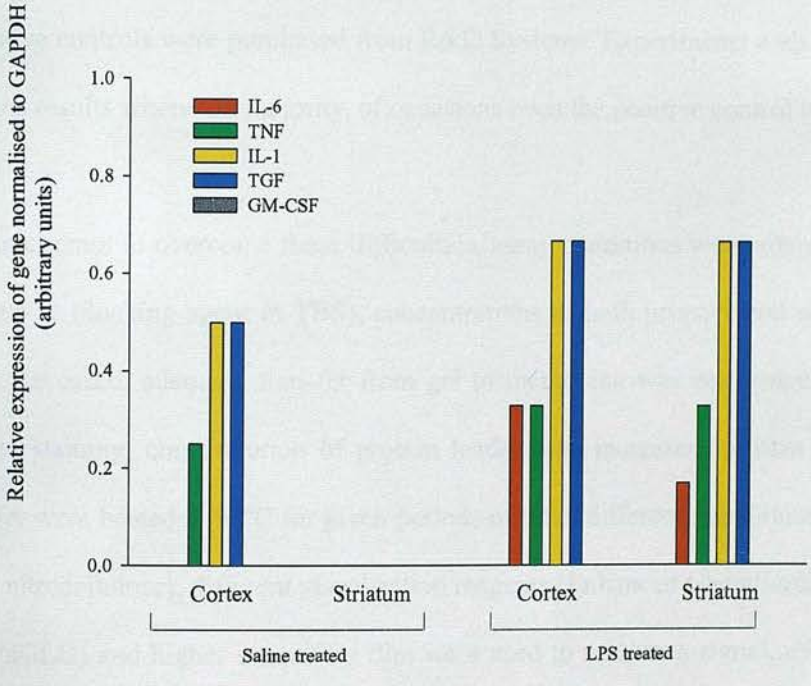


Figure 47. Graphical analysis of MPCR electrophoresis gel showing expression of inflammatory cytokines in the striatum following 3 hrs of LPS stimulation.

CYTOKINE WESTERN ANALYSIS

Having demonstrated changes in cytokine mRNA expression by MPCR following LPS administration and in ischaemic tissue, subsequent studies were performed to determine whether the message (mRNA) detected was translated into protein in the ischaemic brain. However, western analysis experiments looking at cytokine expression produced no conclusive results. The technology for western blotting is well established within the laboratory and was therefore not thought to be the problem with obtaining results. Initial experiments were conducted using antibodies raised against the cytokines supplied by Stephen Poole at the National Institute of Biological Standards and Controls. New antibodies for each cytokine were obtained, together with recombinant protein control samples, but the experiments were still unsuccessful. Fresh secondary antibodies were also purchased with little success. Commercially available antibodies with corresponding recombinant protein positive controls were purchased from R&D Systems. Experiments with these reagents gave mixed results where, on majority, of occasions even the positive control was not apparent.

In an attempt to overcome these difficulties, assay conditions were altered (BSA in place of Blotto as blocking agent in TBS), concentrations of both primary and secondary antibodies was increased, adequate transfer from gel to membrane was confirmed by Coomassie and silver staining, concentration of protein loaded was increased, protein samples in loading buffer were heated at 95°C for given periods of time, different membranes were tried (PDVF and nitrocellulose), different visualisation reagents (Enhanced Chemiluminescence (ECL) vs. LumiGLO) and higher sensitivity film were used to achieve a signal, all with little success. Occasionally bands were seen on the gel for IL-1β at 33 kDa, which may correspond to the precursor protein, but again this was not consistently seen.

CYTOKINE ENZYME-LINKED IMMUNOSORBENT ASSAY

The availabilty of improved ELISA reagents for the measurement of the cytokines of interest in 2000 led to the analysis of IL-1β and TNFα using this technology. Data from an ELISA that discriminated between the pro- and mature forms of the proteins would provide the most conclusive answers to questions regarding the expression and functional status of the proteins following injury. IL-1β reagents for the human isoform of cytokine are available and although not available for the rat isoform, they are currently in development. At the time of the experiments for this thesis, rat IL-6 reagents were not available.

Data from experiments on tissue from MCA occlusion experiments showed an IL-1β response when assayed at 3 and 24 hrs post-insult with a more pronounced response at 24 hrs. The transient model tended to produce a greater response at both time points although not significantly different from the other models (***Figure 50***). The administration of FK506 appeared to have no effect on the expression of IL-1β in either the transient or the permanent model at either time point. Analysis of TNFα expression in the same models suprisingly showed that there was no expression of the cytokine following an ischaemic insult in any model or following any treatment (***Figure 51***). LPS treated tissue was included as a positive control for both IL-1β and TNFα and confirmed the ability to detect increase protein levels following a pro-inflammatory stimulus.

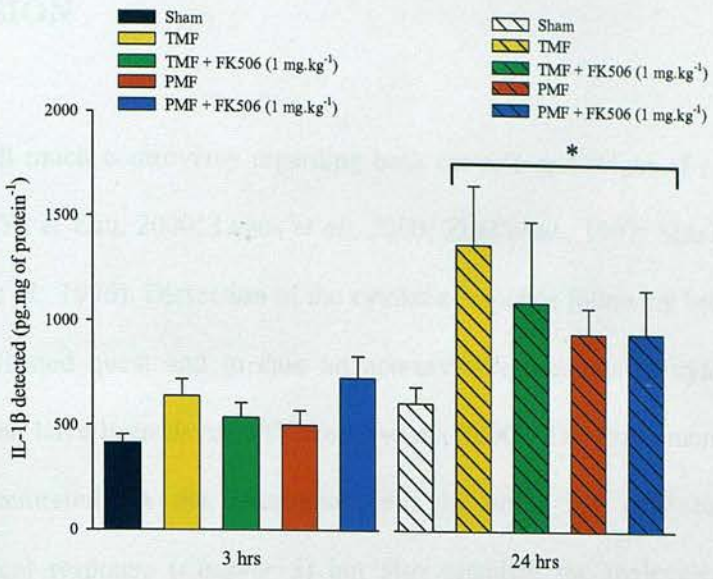


Figure 50. Concentration of IL-1β (pg.mg of protein^{-1}) detected by ELISA following 3 and 24 hrs sham, permanent and 2 hr transient MCA occlusion (n = 3 for each) with and without FK506 treatment. Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test).

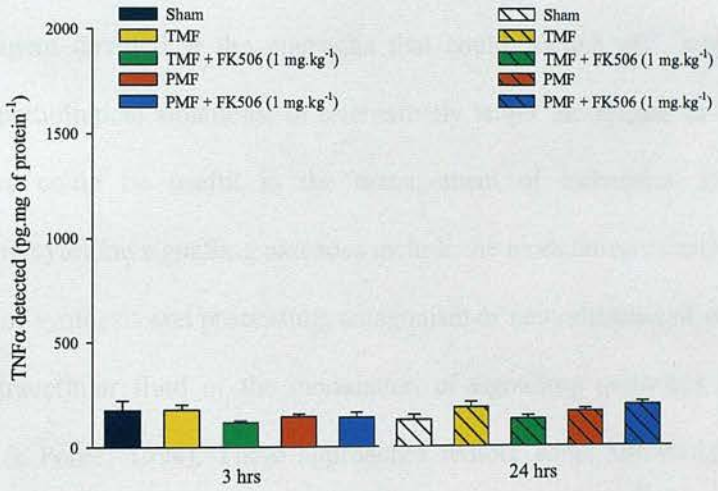


Figure 51. Concentration of TNFα (pg.mg of protein^{-1}) detected by ELISA following 3 and 24 hr sham, permanent and 2 hr transient MCA occlusion (n = 3 for each) with and without FK506 treatment. Data represent mean ± sem. (* $p < 0.05$, 2-way ANOVA with *post hoc* Student-Newman-Keuls test).

DISCUSSION

There is still much controversy regarding both the role and origin of cytokines in cerebral ischaemia (Yu & Lau, 2000; Legos *et al.*, 2000; Zhai *et al.*, 1997; Sharma & Kumar, 1998; Yamasaki *et al.*, 1996). Dissection of the cytokine response following brain injury appears to be a complicated quest and to date no non-invasive methods of cytokine detection and quantification have been devised (Vitkovic *et al.*, 2000). The experiments performed in this thesis concentrated on the microglial cells in order not only to characterise their morphological response (*Chapter 5*) but also establish the molecules they release in a pathological situation and extend this to stroke tissue. As discussed in the previous chapters, microglia respond within minutes of an ischaemic insult and are a potential source of the pro-inflammatory cytokines. They are therefore considered to be deleterious cells. Recently Vitkovic *et al.* (2000) detected IL- 1β immunoreactivity in the human brain and localised expression to the glial cells of the cortex and not the neurones (Vitkovic *et al.*, 2000). A therapeutic agent directed at the microglia that could 'switch off', attenuate the cellular response in pathological situations, or alternatively target the release of molecules such as the cytokines could be useful in the management of ischaemia. Potential points of intervention in cytokine signalling cascades include the modulation of activation, modulation of pathways of synthesis and processing, antagonism or neutralisation of soluble cytokines in blood or extracellular fluid or the modulation of signalling pathways in the target cells (Henderson & Poole, 1994). These approaches require some knowledge of the temporal response of cytokine production following an ischaemic insult. Analysis of gene expression following the manipulation of a biological system is important in terms of characterising the response and provides a starting point for further investigation. It is important however to note that the presence of mRNA does not always imply that the protein coded by the message is present. This strengthens the need for conformation of translated protein in the

tissue by techniques such as western analysis or ELISA. Another consideration regarding protein is whether it is functional, particularly in a disease setting and can be assessed by bioassay.

The analysis of the mRNA for the pro-inflammatory cytokines was initially conducted in LPS stimulated rat brain in order to prove that the technology employed detected the relevant genes of interest and changes therein. Two of the three primers designed and used initially for RT-PCR on LPS stimulated tissue would have been suitable for analysis of the responses in ischaemic tissue once optimal conditions had been established. The availability of the multiplex PCR system however allowed the analysis of a number of genes of interest in one reaction thereby saving tissue, reagents and time. The graphical analysis of the electrophoresis gels although not ideal, allowed the results to be interpreted. Quantification of PCR products can be done using equipment such as a phosphoimager or machines such as Taqman (Applied Biosystems) but at the time these experiments were performed such equipment was not available in the laboratory.

LPS stimulated tissue was again used to prove that the system was able to detect the genes of interest before the analysis of MCA occlusion tissue. LPS administration (3 hrs) caused changes in gene expression in cytokines with the most notable increase in IL-1β, particularly in the striatum. Eriksson *et al.* (2000) suggested that LPS could cause stimulation by either passing through the blood brain barrier and having a direct effect on cells of the parenchyma or by causing the release of the cytokines from microglia which have been activated by factors released from the endothelium (Eriksson *et al.*, 2000). IL-6 and TGF-β were detected in both the cortex and the striatum following LPS administration. Multiplex PCR experiments were also performed on the *in vitro* microglia that had been exposed to LPS for 3 or 24 hrs. The only changes detected at 3 hrs were an increase in IL-6 and GM-CSF

expression. The administration of FK506 to the cultures appeared to have no effect at 3 hrs. At 24 hrs the only detectable change is a reduction in the level of IL-6.

Experiments were conducted on ischaemic tissue from both the transient and permanent monofilament model and on microglial cells in culture. In the initial experiments contralateral hemisphere tissue was included and no changes in expression were noted and therefore only ipsilateral cortical and striatal tissue was analysed in subsequent experiments. Although other researchers have noted changes in gene expression in the contralateral hemisphere, damage was not routinely detected. Changes that can occur contralaterally may be the consequence of physical deformation of the contralateral hemisphere by oedema within the ischaemic hemisphere (Loddick *et al.*, 1998). Although changes contralaterally to the lesion occur, the ipsilateral hemisphere is the main site of ischaemic damage and these studies focused primarily on changes detected in this tissue. This decision is supported by data from Jander *et al.* (2000) who showed that showed no changes in cytokine expression in the contralateral hemisphere using quantitative RT-PCR (Jander *et al.*, 2000).

Analysis of the genes expressed in the cortex of the transient and permanent models and associated shams at 3 and 24 hrs revealed that TNFα was produced at 3 hrs in the transient model but only at 24 hrs in the permanent occlusion model. IL-1β expression in the transient model was greater than both the sham and the permanent occlusion model. These results could suggest that an inflammatory response occurs more rapidly in the transient model, which would be consistent with an endogenous cell response associated with reperfusion (Legos *et al.*, 2000). The greater IL-1β response at 24 hrs in the transient model and delayed expression of TNFα and IL-1β in the permanent model could be associated with an exogenous infiltrating peripheral cell response. In the striatal tissue, both the sham and the transient model showed identical expression of the cytokines of interest at 3 and 24 hrs

although there was no IL-6 expression in either of these experimental groups at 24 hrs. The IL-6 gene expression differs from that seen by other investigators at 24 hrs (Hill *et al.*, 1999). This may not be that surprising as the striatum is the region that does experience the most severe reduction in blood flow following MCA occlusion. Expression in the sham animals is more difficult to explain however the brain is exquisitely sensitive to changes and all experimental procedures do perturb it in some manner which may potentially cause a minor induction or upregulation of the cytokines (Vitkovic *et al.*, 2000). In the permanent model, no cytokines except TGF-β were detected at 3 or 24 hrs. This observation could be explained by absence of cells in the tissue capable of producing the cytokines. It is worth considering that the evolution of the penumbra is a dynamic three-dimensional event and this may be sampled differently in different experimental subjects. The detection of TNFα mRNA at 3 hrs post-insult is consistent with other reports of early detection (Liu *et al.*, 1994). TGF-β was only analysed because it was included in the reagents chosen in order to examine IL-1β, TNFα and IL-6. TGF-β has been suggested to play an anti-inflammatory role in tissue modelling following ischaemia and be involved in tissue remodelling (Lehrmann *et al.*, 1995). Previous studies have showed that it shows a more delayed expression that than IL-1β, TNFα and IL-6 and parallels the accumulation of monocyte/macrophages in the ischaemic cortex (Wang *et al.*, 1995a). The observations here regarding TGF-β suggest that it is expressed as early as 3 hrs. Lehrmann *et al.* (1998) showed by immunohistochemistry, that TGF-β mRNA was localised to the microglia (Lehrmann *et al.*, 1998).

In summary, the analysis of cytokine mRNA expression in these experiments demonstrated that ability to detect the responses described above. The expression of the cytokines following LPS administation was different to that seen in the stroke tissue which is not unsurprising given the different nature of the cellular challenge. For the *in vivo* experiments, tissue from one animal of each experimental group was used in the multiplex PCR

experiments. Ideally more experiments would be performed for each group to verify the observations but the reagents are extremely costly. Pooling tissue was considered but discounted because an overall clear cut result could not be guaranteed. IL-1β expression *in vivo* is stimulated by activation of excitatory amino acid receptors by excitotoxins in a similar fashion to that seen following ischaemia (Pearson *et al.*, 1999). In a recent study, Jander *et al.* (2000) demonstrated for the first time that cytokine induction following ischaemia is by NMDA receptor signalling pathways. This induction occurrs at sites distant from the lesion and was independent of cell death. This observation which strengthen a case for cytokines having a protective role depending on the environment in which they are expressed (Jander *et al.*, 2000). Infusion of IL-1ra with the excitotoxins inhibits brain damage implicating IL-1β in excitotoxic cell death (Rothwell, 1999). IL-1β has highly site-specific neurodegenerative actions in the brain. IL-1β injected into the striatum exacerbates damage in both the cortex and striatum of rats subjected to MCA occlusion but has no effect when administered into the cortex (Stroemer & Rothwell, 1998). Although IL-1α is increased in the brain by stimuli similar to those that induce IL-1β expression it is at lower levels and after the β isoform and it is suggested that IL-1β is the primary mediator of neurodegeneration (Rothwell, 1999).

The present data regarding IL-6 expression differs from that seen by others. In the rat, IL-6 mRNA is detected at 3 hrs after permanent MCA occlusion peaks at 12 hrs and remains high for at least 24 hrs (Wang *et al.*, 1995b). Ali *et al.* (2000) showed upregulation of IL-6 mRNA following ischaemia with no effect on the transcription of its receptors (Ali *et al.*, 2000). It has been reported that IL-6 expression levels are highest in ischaemia reperfusion models (Clark *et al.*, 2000) with a peak in the cortical regions at 24 hrs (Suzuki *et al.*, 2000). The rapid induction of IL-6 suggests that it is produced by the resident cells of the brain and not infiltrating cells (Loddick *et al.*, 1998) although the peak at 24 hrs would suggest circulating

cell contribution. Suzuki *et al.* (1999) demonstrated IL-6 expression in reactive microglia and neurones but stated that the main localisation of the protein was in neurones (Suzuki *et al.*, 1999). IL-6 expression in the ischaemic penumbra occurs at 1 day post insult and is localised to the microglia although neurones have been shown to express the protein under certain pathological conditions (Block *et al.*, 2000). Transgenic animals with the IL-6 gene deleted have an attenuated astrocytic response (assessed by GFAP staining) following an ischaemic insult with a more moderate reduction in microglial activation. In another study, mice lacking IL-6 were no different in terms of infarct size and neurological function when subjected to transient ischaemia. The authors interpreted this observation as IL-6 having no influence on ischaemic injury although the cytokine could have an effect on the long-term recovery following ischaemia. In the IL-6 knockout animals the levels of expression of the other cytokines (IL-1β and TNFα) were less suggesting that IL-6 may enhance the expression of these cytokines via positive feedback mechanisms. This decreased inflammatory response was however not associated with a reduction in ischaemic injury (Clark *et al.*, 2000). Experimentation with transgenic animals has been one method used to study the cytokine function in the pathophysiology of ischaemia however knock out animals have shown that compensatory mechanisms exist and the results should be interpreted with caution (Turrin & Plata-Salaman, 2000). Loddick *et al.* (1998) demonstrated that IL-6 bioactivity increases following permanent MCA occlusion peaking at 24 hrs and proposed a neuroprotective role for the cytokine that has been suggested by others (Beamer *et al.*, 1995; Clark *et al.*, 1999). They also demonstrated that the administration of exogenous IL-6 reduced ischaemic damage (Loddick *et al.*, 1998). Suzuki *et al.* (2000) speculated that activated microglia seen following ischaemia area are a secondary source of IL-6 and may be associated with neuroprotection (Suzuki *et al.*, 2000).

Experiements to detect the presence of protein in LPS stimulated and ischaemic tissue were performed once analysis of cytokine expression at the message level had been established. The failure to achieve any results with the Western analysis was disappointing although it is well recognised that the detection of cytokines by this method is notoriously difficult (Rothwell *et al.*, 1996). A number of different approaches of tissue preparation were used, conditions for incubations altered and detection system changed with little success. Again time constraints limited the pursuit of this technique. ELISA experiments for the cytokines were not used initially because of the inability to distinguish between the pro- and mature forms of the proteins, in particular IL-1β. Recently ELISA assays have been produced that do detect the different forms of the protein and are currently being set up for use in the laboratory. As mentioned previously analysis of IL-6 by ELISA was not performed because at present there are no reagents available. At the time of experimentation the IL-1β reagents for the rat isoforms were not available but were being developed. The experiments with the available reagents were performed with a view to completing the experiments when the improved reagents were obtainable. Legos *et al.* (2000) showed for the first time that the temporal changes in IL-1β protein assessed by ELISA following MCA occlusion were consistent with published data on the upregulation of the cytokine's mRNA. They also demonstrated that the later larger elevation of IL-1β protein at 3 days post-occlusion was associated with the influx of inflammatory cells (Legos *et al.*, 2000).

ELISA experiments were not performed on cultured microglial cells as these studies were being undertaken by Mrs Eliane Chirnside, working on a parallel project. Both IL-1β and TNFα were detected in the ischaemic tissue using the ELISA. LPS was again included as a positive control. The increases in the IL-1β protein mirrored the increase in mRNA detected by MPCR with detection at 3 hrs and a greater response seen at 24 hrs. In contrast, the TNFα data was surprising as the MPCR data showed the presence of mRNA but no protein was

detected at either 3 or 24 hrs. This observation may be explained by inability of the compromised cells in the ischaemic tissue to translate the message into protein. Protein translation requires ATP which may be limited and intact translation machinery that may be damaged following the reduction of blood flow (Sharp *et al.*, 2000; Krause & Tiffany, 1993). Differences in IL-1β and TNFα protein translation may be explained by different sensitivites of the associated enzymes in the ischaemic environment. There were also discrepancies in the data for the permanent monofilament model. Analysis of the mRNA showed an IL-1β and TNFα response at 24 hrs only whereas IL-1β was detected following permanent occlusion at 3 hrs by ELISA and no TNFα was detected at all.

CONCLUSIONS

The main conclusion that can be drawn from the observations concerning the cytokine expression in this chapter is that while there is a notable response, it has been difficult to quantify and assess. The data from the *in vivo* experiments tend to suggest that cytokines are expressed earlier in the transient model and may therefore be an endogenous response whereas the later response in the permanent model would fit with the infiltration of peripheral inflammatory cells. It has also been demonstrated that the cytokine response to LPS is different to that observed following an ischaemic insult. The data shows, not unsurprisingly that dead cells (i.e. those in the ischaemic core) do not produce cytokine mRNA and that the presence of a message does not necessarily equate with protein expression. The microglia do appear to be a source of cytokines following LPS stimulation and the natural extension of these experiments would be to examine the cytokine response following an anoxic insult.

CHAPTER 7

General discussion & summary

The inflammatory response to cerebral ischaemia is a complex interaction diverse of signalling pathways involving the cells of the brain parenchyma, which occurs in a compromised setting for cell survival. The dissection of the components of this response has proven challenging but has been approached in a systematic manner in order to establish the contribution of various elements of the response to the pathophysiology. The flow diagram shows a schematic representation of the thesis rationale (***Figure 52***).

Initial experiments established both the rat permanent and transient monofilament model of MCA occlusion and compared these two models and the Endothelin-1 model of cerebral ischaemia, both in terms of volume of damage and oedema. The data revealed that there were no significant differences in either parameter but that the administration of FK506 showed different neuroprotective efficacy of the compound in the different models. In this respect, FK506 had a greater protective effect in the transient monofilament model. Experiments that examined increasing times of ischaemia suggested that reperfusion was not detrimental to the tissue as no differences were observed in lesion volume. The unequal neuroprotective effect of FK506 in transient and permanent model appeared to reflect differences in tissue response associated with reperfusion and reperfusion-linked differences in the inflammatory response following vessel occlusion. The Endothelin-1 model was not included in further studies although it is considered to be a permanent occlusion (Sharkey *et al.*, 1994). The duration of the ischaemia is uncontrolled and there is a gradual return

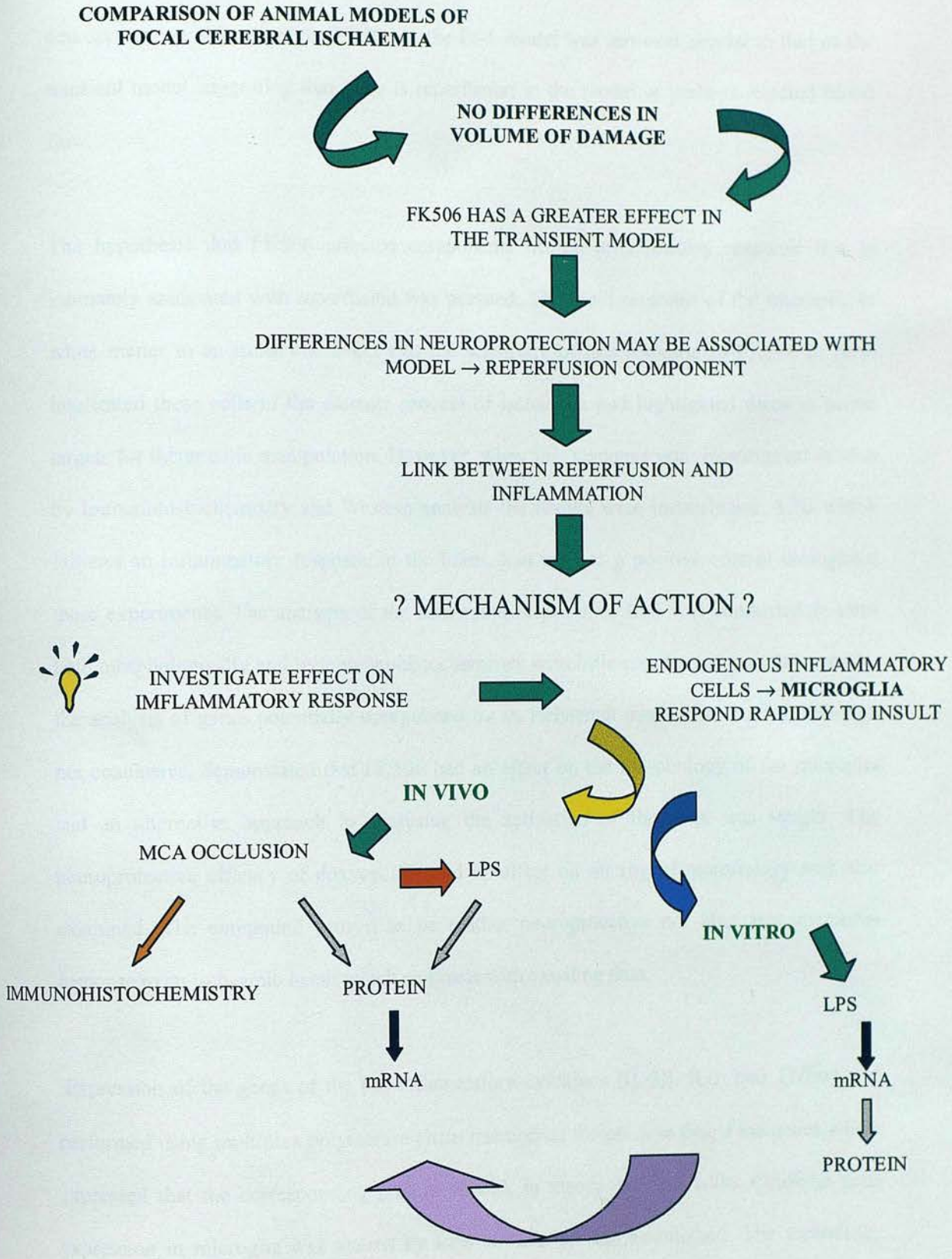


Figure 52. Schematic representation of thesis.

of blood between 3 and 6 hrs post-occlusion which would further complicate analysis. The neuroprotective efficacy of the FK506 in the Et-1 model was however similar to that of the transient model suggesting that there is reperfusion in the model or perhaps residual blood flow.

The hypothesis that FK506 affected components of the inflammatory response that is intimately associated with reperfusion was pursued. The rapid response of the microglia in white matter to an ischaemic event and the sensitvity of this response to FK506 *in vivo*, implicated these cells in the damage process of ischaemia and highlighted them as prime targets for therapeutic manipulation. However, when this response was investigated *in vivo* by immunohistochemistry and Western analysis the results were inconclusive. LPS which initiates an inflammatory response in the brain, was used as a positive control throughout these experiments. The analysis of the microglial response to LPS was conducted *in vitro* both morphologically and by immunohistochemistry with little success and was followed by the analysis of genes potentially upregulated by an ischaemic insult. The studies, although not conclusive, demonstated that FK506 had an effect on the morphology of the microglia and an alternative approach to analysing the activation of the cells was sought. The neuroprotective efficacy of doxycycline and its effect on microglial morphology was also examined. The compound proved to be neither neuroproective nor alter the microglial response to an ischaemic insult which contrasts with exisiting data.

Expression of the genes of the pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) was performed using multiplex polymerase chain reaction on the premise that if the genes where expressed that the corresponding protein should, in theory, be detectable. Cytokine gene expression in microglia was altered by LPS stimulation and established. The technology (MPCR) used was then applied to the tissue from animals that had been subjected to both

permanent and transient MCA occlusion. The initial aim of simplification of the analysis to the gene level was to allow analysis to be conducted in reverse ultimately leading back to the *in vivo* setting. The LPS stimulated expression not only allowed detection and validation of the technology used but showed a different response to that seen following ischaemia. ELISA experiments for IL-1β and TNFα provided information regarding their expression in ischaemic tissue. IL-1β was detected at both 3 and 24 hrs post-insult with a greater response noted at 24 hrs. This expression could be linked to the infiltration of peripheral immune cells such as leukocytes. No TNFα response was detected despite the presence of mRNA. There was no difference in expression of the cytokines between models and FK506 administration had no effect on expression.

The conclusions that can be drawn from the work in this thesis suggest that there is a small but detectable inflammatory response to focal cerebral ischaemia. The microglial data could suggest their involvement in an acute response that may be FK506 sensitive but because the experiments are performed at the limits of the technology, it is difficult to give a definitive answer. The inflammatory response shown by IL-1β expression at 24 hrs is possibly an exogenous response and targeting this may be beneficial but is generally regarded to be outwith the therapeutic window for neuroprotection. There are differences in the cellular response to reperfusion and this involves components of the inflammatory cascade in the different models of focal cerebral ischaemia but the differences do not affect the ultimate fate of the cells. It does however provide avenues for therapeutic intervention. In situations where reperfusion is established the tissue appeared to be more amenable to neuroprotection by FK506 and it is possible that this is associated with the blockade of inflammatory mechanisms.

Much of the analysis conducted during this thesis could now be done differently with the availability of new technology and advances in experimental techniques. New antibodies that distinguish the mature and precursor forms of the cytokine proteins are now obtainable, equipment that allows quantitative analysis of the gene products removing the element of subjective analysis of the experiments and machines that analyse and quantify Western analysis gels are now on the market. The work in this thesis has not only investigated the role of inflammation, specifically the contribution of the microglia but has established models and techniques for studying cerebral ischaemia that have been extended and continue to be investigated by others in the laboratory.

APPENDICES

APPENDIX 1 Histology

PHOSPHATE BUFFERED SALINE (PBS)

14.5 mM sodium chloride (Fisher)

11.5 mM disodium hydrogen orthophosphate (anhydrous) (Fisher)

7.25 mM sodium dihydrogen orthophosphate (anhydrous) (Fisons)

Dissolved in MilliQ (MQ) H_2O. pH to 7.4 by addition of NaOH pellets (Fisher).

4 % PARAFORMALDEHYDE FIX (PFA)

Dissolve 40 g paraformaldehyde (Fischer) per 1000 ml PBS. Add 2 NaOH pellets. Heat to 55-60°C on stirring hotplate until clear. Cool and pH to 7.4 with HCl.

PERIODATE-LYSINE-PARAFORMALDEHYDE (PLP) FIX

Lysine phosphate buffer 0.2 M lysine hydrochloride (Sigma)

0.1 M disodium hydrogen orthophosphate (Fisher)

Mixed together 4:1 to get pH 7.4. Doubled the volume with 100 mM phosphate buffer.

Phosphate buffer 1.48 g.500 ml^{-1} sodium dihydrogen orthophosphate (Fisons)

5.75 g.500 ml^{-1} disodium hydrogen phosphate (Fisher)

4 Paraformaldehyde 8 g.100 ml^{-1} in MQ H_2O (Fisher)

Combined lysine phosphate buffer with paraformaldehyde (3:1) and added 0.2 g.100 ml^{-1} sodium periodate (BDH Chemicals Ltd, UK).

GELATIN SUBBED SLIDES

All slides used for histology were coated with 1 % gelatin (BDH Chemicals Ltd, UK). 5 g of gelatin was dissolved in 500 ml MQ H_2O. Once dissolved 0.5 g chromic potassium sulphate (Sigma) was added and the solution filtered. Slides were immersed in the solution for 2 min and then dried overnight at 37°C.

APPENDIX 2 Cell Culture

COMPLETE BASAL MEDIUM EAGLE (BME)

Basal Medium Eagle (Sigma)

10 % heat-inactivated foetal calf serum (FCS) (46-53°C for 30 min) (GibcoBRL)

0.5 % glucose (8.3 ml of 30 % stock solution) (Sigma)

2 mM L-glutamine (Sigma)

Penicillin-streptomycin (100 U.0.1 mg^{-1}) (Sigma)

Heat inactivated FCS, glucose, glutamine and penicillin-streptomycin were added to 500 ml Basal Medium Eagles and stored at 4°C.

TRYPSIN

12.5 mg dissolved in 4 ml Hank's Balanced Salt Solution (HBSS; Gibco) and then filtered sterilised (Nalgene 0.2 μm syringe filter).

DNASE

DNase (10 000 U; Sigma) was dissolved in 3 ml HBSS (Gibco) and filtered sterilised (Nalgene 0.2 μm syringe filter). 1 ml was added to 4 ml trypsin and 2 ml to 8 ml of the Complete Basal Medium Eagle.

APPENDIX 3 Molecular Biology

10x RNA GEL RUNNING BUFFER

266 mM MOPS (Sigma)

2.48 g ethylenediamine tetra-acetic acid (EDTA) disodium salt (Fisher)

2.73 g Sodium acetate (BDH Chemicals)

Made up to 500 ml with MQ H_2O. pH 7.0 with 5 M NaOH (Fisher) and stored at 4°C

'LARGE' FORMALDEHYDE AGAROSE GEL ELECTROPHORESIS

1.5 % AGAROSE IN 1 X RUNNING BUFFER

2.25 g agarose (Promega) melted in 127.5 ml MQ H_2O in the microwave.15 ml 10x RNA gel running buffer added and allowed to cool to < 50°C. 8.1 ml formaldehyde (Sigma) added, gel poured and allowed to set.

'SMALL' FORMALDEHYDE AGAROSE GEL ELECTROPHORESIS

1.5 % Agarose in 1 x Running Buffer

0.563 g agarose (Promega) melted in 31.9 ml MQ H_2O in the microwave. 3.75 ml 10x RNA gel running buffer added and allowed to cool to < 50°C. 2.025 ml formaldehyde (Sigma) added, gel poured and allowed to set.

RNA SAMPLE BUFFER

100 µl 10x RNA gel running buffer

178 µl formaldehyde (Sigma)

500 µl formamide (Promega)

RNA DYE SOLUTION

0.1 % Bromophenol Blue (Sigma)

7.5 % Ficoll (Sigma)

MASTER MIX

For each PCR reaction a master mix including the upstream and downstream primers for the gene of interest was made in a final reaction volume of 50 µl.

1 x PCR buffer (Promega)

2 mM $MgCl_2$ (Promega)

200 nM dNTPs (deoxyribonucleoside triphosphates, Pharmacia)

0.5 µM of upstream and downstream primers (SigmaGenosys)

2.5U Taq polymerase (Promega)

MQ H_2O

50X TRIS ACETATE EDTA (TAE) BUFFER

242 g Tris base (Sigma)

57.1.ml glacial acetic acid (Fisher)

100 ml 0.5 M EDTA (Sigma) pH 8

AGAROSE GEL ELECTROPHORESIS

0.8 % agarose (Promega) gel for routine electrophoresis and 2 % gel for electrophoresis of MPCR products in 1 x TAE (0.4 M Tris acetate; 0.001 M EDTA, Sigma)

0.8 or 2 g agarose melted with 2 ml 50x TAE and topped up to 100 ml with MQ H_2O. Allowed to cool to < 50°C, 3 µl ethidium bromide (EtBr, Sigma; 50 $mg.ml^{-1}$) added, poured and allowed to set.

MULTIPLEX POLYMERASE CHAIN REACTION (MPCR) MIXTURE

Reagents supplied with MPCR detailed in *Chapter 6*.

30.5 µl	distilled water
5 µl	10x MPCR buffer
5 µl	10x MPCR primers
0.5 µl	*Taq* DNA polymerase (Promega)
5 µl	Sample cDNA or 10x control cDNA from kit
4 µl	3.12 mM dNTP

Nucleotide sequence of interleukin-1β (Acc. # E01884). The derived amino acid sequence is shown above the nucleotide sequence. Nucleotides are numbered on the left starting from the A of the ATG initiation codon. The green boxes denoted the binding sites of the upstream and downstream primers for a 522 bp PCR fragment (Delgado-Rizo *et al.*, 1998).

GGCATAACAGGCTCATCTGGGATCCTCTCCAGTCAGGCTTCCTTGTGCAAGTGTCTGAAGCAGCT

Met Ala Thr Val Pro Glu Leu Asn Cys Glu Ile Ala Ala Phe Asp Ser Glu 17

1 ATG GCA ACT GTC CCT GAA CTC AAC TGT GAA ATA GCA GCT TTC GAC AGT GAG

Glu Asn Asp Leu Phe Phe Glu Ala Asp Arg Pro Gln Lys Ile Lys Asp Cys 34

66 GAG AAT GAC CTG TTC TTT GAG GCT GAC AGA CCC CAA AAG ATT AAG GAT TGC

Phe Gln Ala Leu Asp Leu Gly Cys Pro Asp Glu Ser Ile Gln Leu Gln Ile 51

117 TTC CAA GCC CTT GAC TTG GGC TGT CCA GAT GAG AGC ATC CAG CTT CAA ATC

Ser Gln Gln His Leu Asp Lys Ser Phe Arg Lys Ala Val Ser Leu Ile Val 68

168 TCA CAG CAG CAT CTC GAC AAG AGC TTC AGG AAG GCA GTG TCA CTC ATT GTG

Ala Val Glu Lys Leu Trp Gln Leu Pro Met Ser Cys Pro Trp Ser Phe Gln 85

219 GCT GTG GAG AAG CTG TGG CAG CTA CCT ATG TCT TGC CCG TGG AGC TT[redacted]

Asp Glu Asp Pro Ser Thr Phe Phe Ser Phe Ile Phe Glu Glu Glu Pro Val 102

270 [redacted]CC TTC TTT TCC TTC ATC TTT GAA GAA GAG CCC GTC

Leu Cys Asp Ser Trp Asp Asp Asp Asp Leu Leu Val Cys Asp Val Pro Ile 119

321 CTC TGT GAC TCG TGG GAT GAT GAC GAC CTG CTA GTG TGT GAT GTT CCC ATT

Arg Gln Leu His Cys Arg Leu Arg Asp Glu Gln Gln Lys Cys Leu Val Leu 136

372 AGA CAG CTG CAC TGC AGG CTT CGA GAT GAA CAA CAA AAA TGC CTC GTG CTG

Ser Asp Pro Cys Glu Leu Lys Ala Leu His Leu Asn Gly Gln Asn Ile Ser 153

423 TCT GAC CCA TGT GAG CTG AAA GCT CTC CAC CTC AAT GGA CAG AAC ATA AGC

Gln Gln Val Val Phe Ser Met Ser Phe Val Gln Gly Glu Thr Ser Asn Asp 170

474 CAA CAA GTG GTA TTC TCC ATG AGC TTT GTA CAA GGA GAG ACA AGC AAC GAC

Lys Ile Pro Val Ala Leu Gly Leu Lys Gly Lys Asn Leu Tyr Leu Ser Cys 187

525 AAA ATC CCT GTG GCC TTG GGC CTC AAG GGG AAG AAT CTA TAC CTG TCC TGT

Val Met Lys Asp Gly Thr Pro Thr Leu Gln Leu Glu Ser Val Asp Pro Lys 204

576 GTG ATG AAA GAC GGC ACA CCC ACC CTG CAG CTG GAG AGT GTG GAT CCC AAA

Gln Tyr Pro Lys Lys Lys Met Glu Lys Arg Phe Val Phe Asn Lys Ile Glu 221

627 CAA TAC CCA AAG AAG AAG ATG GAA AAG CGC TTT GTC TTC AAC AAG ATA GAA

Val Lys Thr Lys Val Glu Phe Glu Ser Ala Gln Phe Pro Asn Trp Tyr Ile 238

678 GTC AAG ACC AAA GTG GAG TTT GAG TCT GCA CAG TTC CCC AAC TGG TAC ATC

Ser Thr Ser Gln Ala Glu His Arg Pro Val Phe Leu Gly Asn Ser Asn Gly 255

729 AGC ACC TCT CAA GCA GAG CAC AGA CCT GTC TTC CTA [redacted]

Arg Asp Ile Val Asp Phe Thr Met Glu Pro Val Se Ser Stop 268

780 [redacted]C ATA GTT GAC TTC ACC ATG GAA CCC GTG TCT TCC TAA AGATGGCTGCA

833 CTATTCCTAATGCCTTCCCCAGGACATGCTAGGGAGCCCCCTTGTCGAGAATGGGCAGTCTCCAGGG

900 GAAGCCTTTGTCCTCTGCCAAGTCAGGTCTCTCAGAGCCATAAGAAAACCGTGGCACATTCTGGTCA

Nucleotide sequence of interleukin-6 (Acc. # M26744). The derived amino acid sequence is shown above the nucleotide sequence. Nucleotides are numbered on the left starting from the A of the ATG initiation codon. The red boxes denoted the binding sites of the upstream and downstream primers for a 496 bp PCR fragment (Delgado-Rizo *et al.*, 1998).

```
1     AGCTCATTCTGTCTCGAGCCCACCAGGAACGAAAGTCAACTCCATCTGCCCTTCAGGAACAGCT
      Met Lys Phe Leu Ser Ala Arg Asp Phe Gln Pro Val Ala Phe Leu Gly Leu    17
65    ATG AAG TTT CTC TCC GCA AGA GAC TTC CAG CCA GTT GCC TTC TTG GGA CTG
      Met Leu Leu Thr Ala Thr Ala Phe Pro Thr Ser Gln Val Arg Arg Gly Asp    34
116   ATG TTG TTG ACA GCC ACT GCC TTC CCT ACT TCA CAA GTC CGG AGA GGA GAC
      Phe Thr Glu Asp Thr Thr His Asn Arg Pro Val Tyr Thr Thr Ser Gln Val    51
167   TTC ACA GAG GAT ACC ACC CAC AAC AGA CCA GTA TAT ACC ACT TCA CAA GTC
      Gly Gly Leu Ile Thr Tyr Val Leu Arg Glu Ile Leu Glu Met Arg Lys Glu    68
218   GGA GGC TTA ATT ACA TAT GTT CTC AGG GAG ATC TTG GAA ATG AGA AAA GAG
      Leu Cys Asn Gly Asn Ser Asp Cys Met Asn Ser Asp Asp Ala Leu Ser Glu    85
269   TTG TGC AAT GGC AAT TCT GAT TGT ATG AAC AGC GAT GAT GCA CTG TCA GAA
      Asn Asn Leu Lys Leu Pro Glu Ile Gln Arg Asn Asp Gly Cys Phe Gln Thr   102
320   AAC AAT CTG AAA CTT CCA GAA ATA CAA AGA AAT GAT GGA TGC TTC CAA ACT
      Gly Tyr Asn Gln Glu Ile Cys Leu Leu Lys Ile Cys Ser Gly Leu Leu Glu   119
371   GGA TAT AAC CAG GAA ATT TGC CTA TTG AAA ATC TGC TCT GGT CTT CTG GAG
      Phe Arg Phe Tyr Leu Glu Phe Val Lys Asn Asn Leu Gln Asp Asn Lys Lys   136
422   TTC CGT TTC TAC CTG GAG TTT GTG AAG AAC AAC TTA CAA GAT AAC AAG AAA
      Asp Lys Ala Arg Val Ile Gln Ser Asn Thr Glu Thr Leu Val His Ile Phe   153
473   GAC AAA GCC AGA GTC ATT CAG AGC AAT ACT GAA ACC CTA GTT CAT ATC TTC
      Lys Gln Glu Ile Lys Asp Ser Tyr Lys Ile Val Leu Pro Thr Pro Thr Ser   170
524   AAA CAA GAG ATA AAA GAC TCA TAT AAA ATA GTC CTT CCT ACC CCA ACT TCC
      Asn Ala Leu Leu Met Glu Lys Leu Glu Ser Gln Lys Glu Trp Leu Arg Thr   187
575   AAT GCT CTC CTA ATG GAG AAG TTA GAG TCA CAG AAG GAG TGG CTA AGG ACC
      Lys Thr Ile Gln Leu Ile Leu Lys Ala Leu Glu Glu Phe Leu Lys Val Thr   204
626   AAG ACC ATC CAA CTC ATC TTG AAA GCA CTT GAA GAA TTT CTA AAG GTC ACT
      Met Arg Ser Thr Arg Gln Thr Stop                                     211
677   ATG AGG TCT ACT CGG CAA ACC TAG TGTGCTATGCCTAAGCATATCAGTTTGTGGACATT
736   CCTCACTGTGGTCAGAAAATATATCCTGTCGATGGGTATCTAAATTATGTTGTTCTCTACGAAGAAC
803   TGGCAATATGAATGTTGAAACACTATTTTAATTATTTTTAATTTATTGATAATTTAAATAAGTAAAC
870   TATAAGTTAATTTATGATTGATATTTATACTTTTTATGAAGTGTCACTTGAAATATTATGTTATAGT
937   TTTGAAAAGATAATATAAAAATCTATTTGATATGAATATTCTCTTACCTAGCCAGATGGTTTCTTGC
1004  AATATATAAGTTTACCTCAATGAATTGCTAATTTAAATTTTTT
```

Nucleotide sequence of tumour necrosis factor α (Acc. # X66539/S40199). The derived amino acid sequence is shown above the nucleotide sequence. Nucleotides are numbered on the left starting from the A of the ATG initiation codon. The yellow boxes denoted the binding sites of the upstream and downstream primers for a 468 bp PCR fragment (Delgado-Rizo *et al.*, 1998).

```
    Met Ser Thr Glu Ser Met Ile Arg Asp Val Glu Leu Ala Glu Glu Ala Leu     17
  1 ATG AGC ACA GAA AGC ATG ATC CGA GAT GTG GAA CTG GCA GAG GAG GCG CTC
    Pro Lys Lys Met Gly Gly Leu Gln Asn Ser Arg Arg Cys Leu Cys Leu Ser     34
 52 CCC AAA AAG ATG GGG GGC CTC CAG AAC TCC AGG CGG TGT CTG TGC CTC AGC
    Leu Phe Ser Phe Pro Leu Val Ala Gly Ala Thr Thr Leu Phe Cys Leu Leu     51
103 CTC TTC TCA TTC CCG CTC GTG GCG GGG GCC ACC ACG CTC TTC TGT CTA CTG
    Asn Phe Gly Val Ile Gly Pro Asn Lys Glu Glu Lys Phe Pro Asn Gly Leu     68
154 AAC TTC GGG GTG ATC GGT CCC AAC AAG GAG GAG AAG TTC CCA AAT GGG CTC
    Pro Leu Ile Ser Ser Met Ala Gln Thr Leu Thr Leu Arg Ser Ser Ser Gln     85
205 CCT CTC ATC AGT TCC ATG GCC CAG ACC CTC ACA CTC AGA TCA TCT TCT CAA
    Asn Ser Ser Asp Lys Pro Val Ala His Val Val Ala Asn His Gln Ala Glu    102
256 AAC TCG AGT GAC AAG CCC GTA GCC CAC GTC GTA GCA AAC CAC CAA GCA GAG
    Glu Gln Leu Glu Trp Leu Ser Gln Arg Ala Asn Ala Leu Leu Ala Asn Gly    119
307 GAG CAG CTG GAG TGG CTG AGC CAG CGT GCC AAC GCC CTC CTG GCC AAT GGC
    Met Asp Leu Lys Asp Asn Gln Leu Val Val Pro Ala Asp Gly Leu Tyr Leu    136
358 ATG GAT CTC AAA GAC AAC CAA CTG GTG GTA CCA GCA GAT GGG CTG TAC CTT
    Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Asp Tyr Val Leu    153
409 ATC TAC TCC CAG GTT CTC TTC AAG GGA CAA GGC TGC CCC GAC TAT GTG CTC
    Leu Thr His Thr Val Ser Arg Phe Ala Thr Ser Tyr Gln Glu Lys Val Ser    170
460 CTC ACC CAC ACC GTC AGC CGA TTT GCC ACT TCA TAC CAG GAG AAA GTC AGC
    Leu Leu Ser Ala Ile Lys Ser Pro Cys Pro Lys Asp Thr Pro Glu Gly Ala    187
511 CTC CTC TCC GCC ATC AAG AGC CCT TGC CCT AAG GAC ACC CCT GAG GGA GCT
    Glu Leu Lys Pro Trp Tyr Glu Pro Met Tyr Leu Gly Gly Val Ser Gln Leu    204
562 GAG CTC AAG CCC TGG TAT GAG CCC ATG TAC CTG GGA GGA GTC TCC CAG CTG
    Glu Lys Gly Asp Leu Leu Ser Ala Glu Val Asn Leu Pro Lys Tyr Leu Asp    221
613 GAG AAG GGG GAC CTG CTC AGC GCT GAG GTC AAC CTG CCC AAG TAC TTA GAC
    Ile Thr Glu Ser Gly Gln Val Tyr Phe Gly Val Ile Ala Leu                235
664 ATC ACG GAG TCC GGG CAG GTC TAC TTT GGA GTC ATT GCT CTG TGA
```

APPENDIX 4 Extraction

EXTRACTION BUFFER

10 mM HEPES KOH pH 7.4 (Sigma)

2 mM $MgCl_2$ (Sigma)

5 mM EGTA (Sigma)

0.5 % CHAPS (Sigma)

50 mM KCl (Sigma)

Phosphatase inhibitor cocktail – 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate

1 mM sodium orthovanadate (Sigma)

10 $\mu g.ml^{-1}$ protease inhibitor cocktail – leupeptin, pepstatin A and aprotinin (Sigma)

1 mM PMSF (Sigma)

10 x extraction buffer minus inhibitor cocktails and PMSF was made, aliquotted and stored at -70°C. Immediately prior to use, 1 x solution was made with inhibitor cocktails and PSMF added.

APPENDIX 5 *Western Blotting*

SDS ACRYLAMIDE GELS

12 % Acrylamide Gel

8 ml 30 % Acrylamide/Bis solution 29:1 (3.3 % C) (BioRad)

4 ml 1.5 M Tris pH 8.8 (Sigma)

0.16 ml 10 % SDS (Sigma)

16 µl TEMED (Sigma)

120 µl 10 % ammonium persulphate (Sigma)

3.7 ml MQ H_2O

4 % Acrylamide Stacking Gel

1.3 ml 30 % Acrylamide/Bis solution 29:1 (3.3 % C) (BioRad)

1.25 ml 1 M Tris pH 6.8 (Sigma)

0.1 ml 10 % SDS (Sigma)

0.2 10 µl TEMED (Sigma)

75 µl 10 % ammonium persulphate (Sigma)

7.3 ml MQ H_2O

For both 12 % and 4 % gels stock solutions for acrylamide, Tris and SDS were made and stored at 4°C. To pour the gels the appropriate volume of TEMED and freshly made ammonium persulphate was added. Gels were poured between Hoefer glass plates (Pharmacia) plates with 10/15 well comb.

SAMPLE BUFFER

2 ml Glycerol (Sigma)

2 ml 10 % SDS (Sigma)

0.25 mg Bromophenol Blue (Sigma)

2.5 ml stacking gel buffer (6.06 g Tris in 100 ml, 4 ml 10 % SDS pH 6.8)

0.5 ml β-mercaptoethanol (Sigma)

STOCK SOLUTIONS OF RECOMBINANT CYTOKINE STANDARDS

Interleukin-1β (R & D Systems, Lot Q202904)

10 mg supplied was reconstituted in 0.5 ml sterile PBS containing 1.5 $mg.ml^{-1}$ BSA (0.15 %) to give 20 $\mu g.ml^{-1}$ stock solution. Aliquotted and stored at -70°C

Interleukin-6 (R & D Systems, Lot ANW01905C)

10 mg supplied in 144 µl 45 % CH_3CN, 0.1 % TFA and 50 µg BSA per 1 µg of cytokine at final concentration of 0.0694 $\mu g.ml^{-1}$. Aliquotted and stored at -70°C.

Tumour Necrosis Factor α (R & D Systems, Lot AGM019051C)

10 mg supplied was reconstituted in 0.5 ml sterile PBS containing 1.5 $mg.ml^{-1}$ BSA (0.15 %) to give 20 $\mu g.ml^{-1}$ stock solution. Aliquotted and stored at -70°C

CYTOKINE ANTIBODIES

Anti - Interleukin-1β (R & D Systems, Lot YR39041)

Anti- Interleukin-6 (R & D Systems, Lot BCZ028102)

Anti-Tumour Necrosis Factor α (R & D Systems, Lot XI028121)

All cytokine antibodies were reconstituted in sterile PBS to give 0.1 $mg.ml^{-1}$ solution, aliquotted and stored at -70°C.

TRIS/GLYCINE BUFFER (10X)

0.25 mM Tris (Sigma)

1.92 M Glycine (Sigma)

RUNNING BUFFER

1 x Tris/glycine buffer

0.1 % SDS (Sigma)

TRANSFER BUFFER

1 x Tris/glycine buffer

20 % methanol (Fisher)

TRIS BUFFERED SALINE-TWEEN 20 (TBST) pH 7.4

50 mM Tris (Sigma)

0.5 M NaCl (Sigma)

0.5 % Tween 20 (Sigma)

SILVER STAINING OF POLYACRYLAMIDE GELS

Gels were initially fixed in 100 ml of 10 % ethanol (v/v) and 0.5 % (v/v) glacial acetic acid for 10 min and then stained in 11.2 mM $AgNO_3$ for 10 min. Gels were washed in MQ H_2O and protein bands developed by addition of 0.75 M NaOH containing 0.25 % formaldehyde. The reaction was stopped after 10 min when the bands were clearly visible by the addition of 1 % glacial acid.

Coomasie sensitivity:	0.1-1 μg
Silver staining sensitivity:	1-10 ng

APPENDIX 6 ELISA

All reagents and samples were brought to room temperature before use.

IL-1β (RLB00)

Assay Diluent RD1-21

Calibrator Diluent RD5Y

IL-1β Standard: Reconstituted with Calibrator Diluent to produce a 2000 $pg.ml^{-1}$ stock solution from which a series of six 2-fold dilutions were made to establish a standard curve.

TNFα (RTA00)

Assay Diluent RD1-41

Calibrator Diluent RD5-17

TNFα Standard: Reconstituted with Calibrator Diluent to produce a 800 $pg.ml^{-1}$ stock solution from which a series of six 2-fold dilutions were made to establish a standard curve.

APPENDIX 7 *Exclusion Criteria*

An animal will be considered to be integral to the study at the origin of the procedure. Exclusion from the study will occur if the animal fulfils any of the following criteria:

a) Death

i) During or post surgery.

ii) Animal terminated by researcher/other due to falling outwith project severity limits.

iii) Animal terminated due to unseen circumstances unrelated to the study that may bias the result (details to be provided).

b) Surgical misses

i) No damage (clear miss determined by histology).

ii) Where no cortical damage is observed and striatal damage is restricted to ≤ 2 of the 9 template regions and < 10 mm^3 and Evans Blue staining confirms incorrect placement of the monofilament.

c) *Standard deviations*

i) Animals will be excluded from the study if upon termination of the study the value of the total volume of damage of an individual animal is > 2 standard deviations from the mean total of the whole group.

d) Other

i) Any contralateral damage.

ii) Reperfusion established > ± 10 min from designated occlusion time.

iii) Drug/vehicle not administered by appropriate route.

iv) Drug/vehicle administered in inappropriate solution.

v) Haematoma/ blood clots observed on brain surface or in blood vessels (i.e. Circle of Willis).

vi) Weight loss > 33 % of pre-operation weight.

The entire study will be excluded if the animal exclusion rate is > 40 %.

APPENDIX 8 *Computerised digital morphometry*

Templates were modified from 'The Rat Brain in Stereotaxic Co-ordinates', Paxinos & Watson (1986) (Paxinos & Watson, 1986). The 9 templates were chosen to cover the area of a characteristic MCA occlusion damage from +4.2 to –7.8 from Bregma (+4.2, +2.2, +1.2, +0.2, -0.8, -3.8, -5.8, -7.8) with easily recognisable anatomical landmarks. The levels were roughly based on those already in use by other investigators who demonstrated accurate assessment of the volume of damage with 8 levels without a loss of accuracy (Osborne *et al.*, 1987). This method of calculating the volume of damage also accounts for distortion or shrinkage that may occur following perfusion fixation and oedema associated with the lesion.

Stained sections were examined by light microscopy and the damage annotated onto the templates in pencil initially and then coloured dark blue. The templates were digitised using the MCID M5+ image analyser (version 4.00 Rev 1.5) and the total area of damage computed for 82 regions of the rat brain (37.7 pixels.mm^{-1} on the horizontal and 36.6 pixels.mm^{-1} on the vertical). Values obtained were transposed into an Excel workbook and the volume of damage calculated using the trapezoid rule (*Chapter 2*). This model has been reported to give consistently accurate results when calculating the volume of objects with unequal distances between sampled sections. A number of other methodologies (rectangular estimation, Cavalieri's rule, Simpson's rule, parabolic estimation) were also compared in the process of establishing the validity of the trapezoid rule for calculation of the volume damage (Rosen & Harry, 1990). Damage calculated was grouped into cortex, striatum and mid-line (all non-cortical and non-striatal regions) regions. Data was graphed using SigmaPlot 5.0 and analysed statistically using SigmaStat 2.03.

VALIDATION

A number of experiments were performed to validate the use of the computerised digital morphometry to quantify the volume of damage. The initial experiment compared observer variation (John Sharkey and Jennifer McCarter). The second experiment compared the volume of damage scored on the new templates with that calculated by the 'cut and weigh' method previously used in the laboratory. There was no significant difference between the two people scoring in terms of volume or distribution of damage (correlation coefficient = 0.95; $n = 17$). Comparison of the new and old methods revealed that was no difference in the volume of damage calculated by the two methods ($p > 0.05$). The new MCID method was however more practical and reproducible and quicker and allows efficient handling of the data generated thus speeding up the time taken to fully analyse a series of experiments.

RAT BRAIN REGIONS

BREGMA +4.2

1. Medial, ventral & ventrolateral orbital cortex; cingulate cortex areas 1 & 3
2. Frontal cortex area 2
3. Agranular insular cortex
4. Lateral orbital cortex
5. Anterior olfactory nuclei

BREGMA +2.2

6. Frontal cortex area 2
7. Frontal cortex area 1 & 3
8. Parietal cortex area 1
9. Agranular insular cortex; ventrolateral & lateral orbital cortex; claustrum
10. Piriform cortex
11. Basal forebrain
12. Accumbens nucleus
13. Caudate putamen
14. Cingulate cortex areas 1 & 3, infralimbic cortex, dorsal peduncular cortex, tenia tecta

BREGMA +1.2

15. Cingulate cortex areas 1 & 2
16. Frontal cortex area 2
17. Frontal cortex area 1; forelimb area of cortex
18. Parietal cortex area 1
19. Agranular, dysgranular & granular insular cortex; claustrum
20. Piriform cortex
21. Septum; basal forebrain
22. Accumbens nucleus
23. Caudate putamen

BREGMA +0.2

24. Cingulate cortex areas 1 & 2
25. Frontal cortex area 2
26. Frontal cortex area 1; forelimb area of cortex
27. Parietal cortex area 1 & 2
28. Agranular, dysgranular & granular insular cortex; claustrum
29. Piriform cortex; dorsal endopiriform cortex
30. Lateral septum; basal forebrain
31. Caudate putamen
32. Medial septum

BREGMA –0.8

33. Cingulate cortex areas 1 & 2
34. Frontal cortex area 2
35. Frontal cortex area 1; forelimb & hindlimb area of cortex
36. Parietal cortex area 1 & 2
37. Agranular & granular insular cortex; claustrum
38. Piriform cortex
39. Anterior hypothalamic area
40. Globus pallidus
41. Caudate putamen

BREGMA –1.8

42. Retrospenial cortex
43. Frontal cortex area 2
44. Frontal cortex area 1; hindlimb area of cortex
45. Parietal cortex area 1 & 2
46. Perirhinal cortex
47. Piriform cortex
48. Hypothalamic nuclei & amygdaloid nuclei
49. Thalamic nuclei
50. Globus pallidus
51. Caudate putamen

BREGMA –3.8

52. Retrospenial cortex
53. Occipital cortex
54. Parietal cortex area 1 & 2
55. Temporal cortex
56. Perirhinal cortex
57. Piriform cortex
58. Amygdaloid nuclei
59. Caudate putamen
60. Cerebral peduncle
61. Hypothalamic nuclei
62. Thalamic nuclei
63. Hippocampus
64. Hanbenula nucleus

BREGMA –5.8

65. Retrospenial cortex
66. Occipital cortex
67. Temporal cortex
68. Perirhinal cortex
69. Entorhinal cortex
70. Hippocampus & amygdaloid nuclei
71. Deep mesencephalic nuclie
72. Substantia nigra
73. Central gray

BREGMA –7.8

74. Retrospenial cortex
75. Occipital cortex
76. Temporal cortex
77. Perirhinal cortex
78. Entorhinal cortex
79. Tegmental nuclei
80. Central grey
81. Raphe nuclei
82. Paralemniscal nuclei

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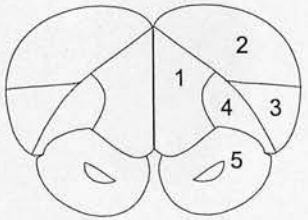

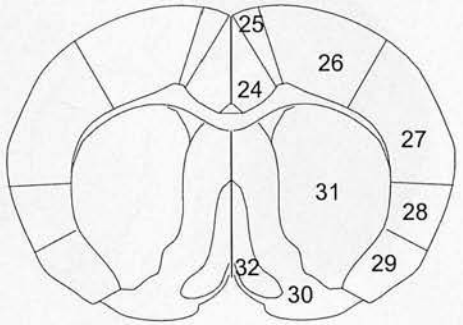

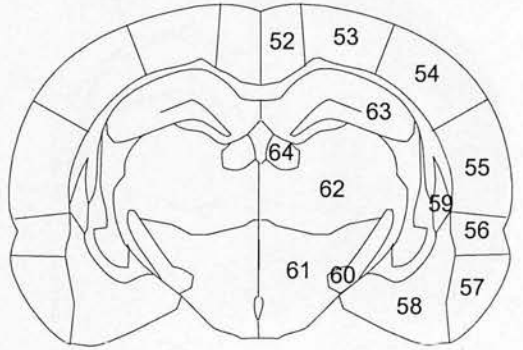

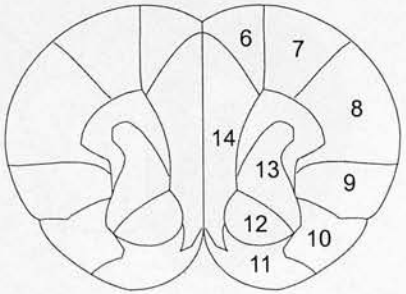

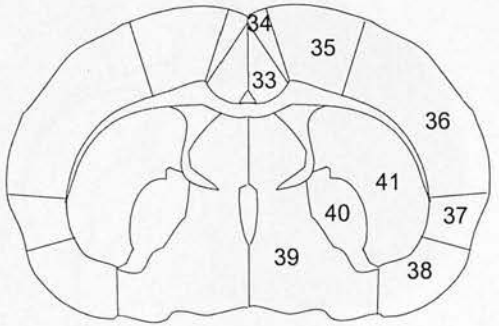

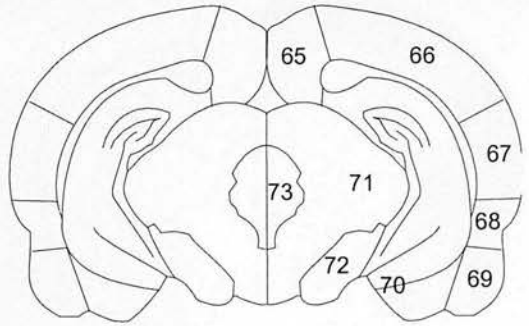

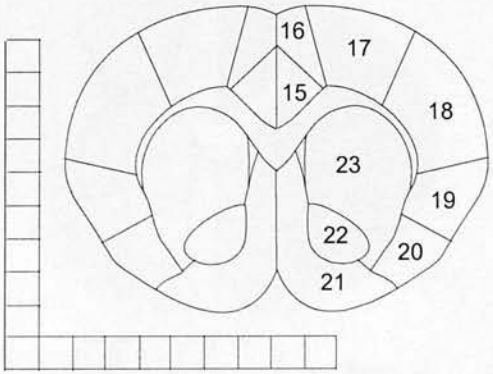

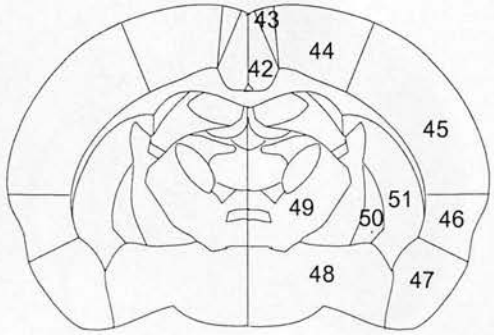

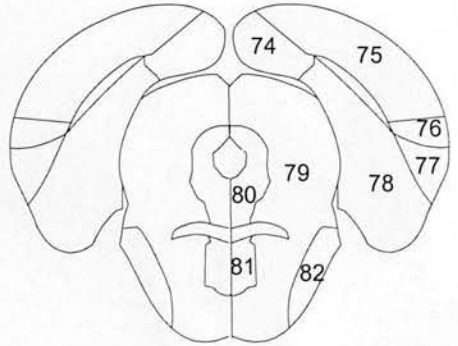

RAT STEREOTACTIC LEVELS: Bregma +4.2; +2.2; +1.2; +0.2; -0.8; -1.8; -3.8; -5.8; -7.8

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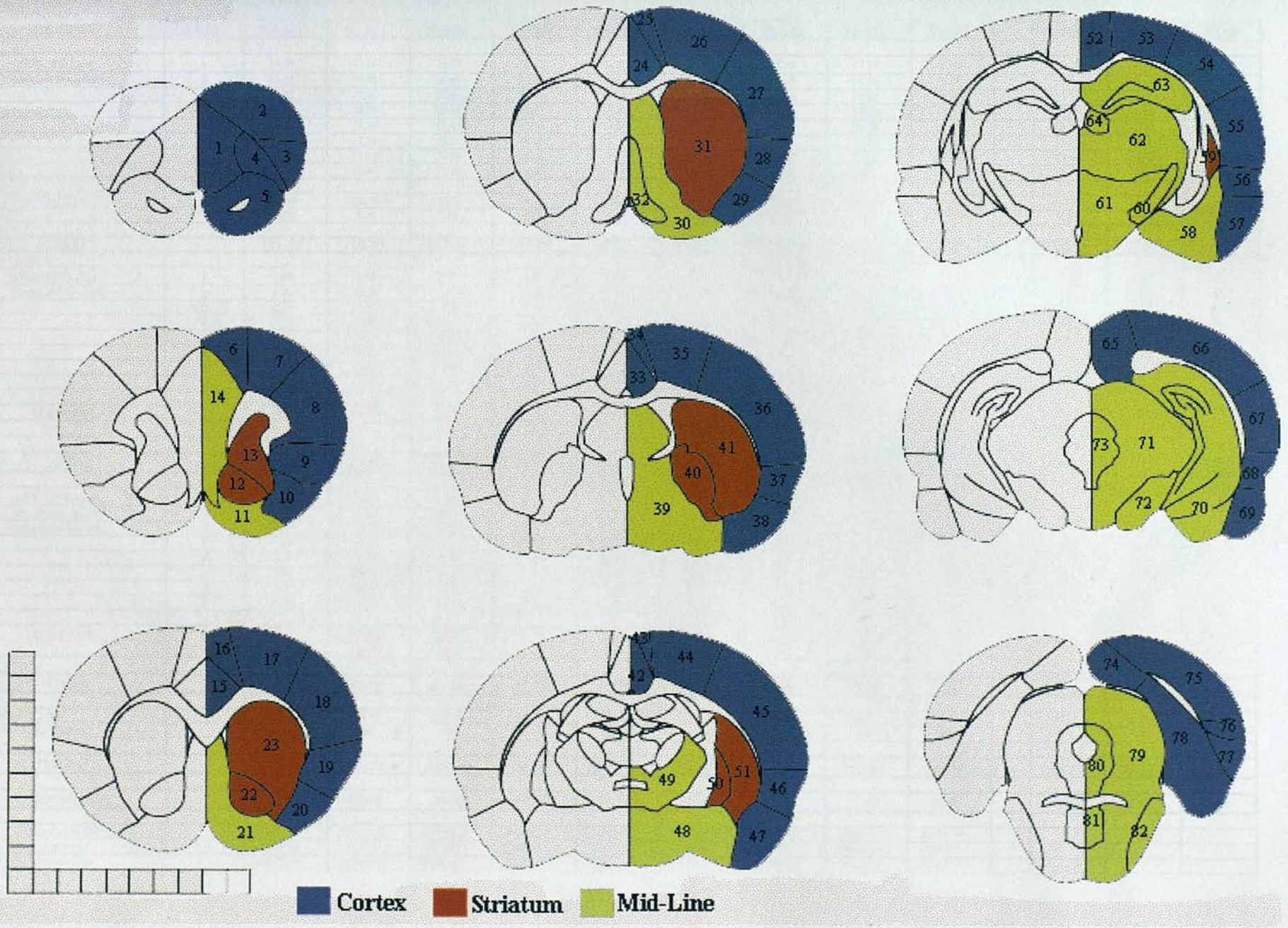
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Cortex
Striatum
Mid-Line

PERMAMENT MONOFILAMENT OCCLUSION

REGION	LEVEL	98-601	98-603	98-606	98-607	98-611	98-642	98-648	98-651	98-662		MEAN	SD	SEM
CORTEX (mm ^{3})	1	14.68	23.16	26.23	32.12	5.95	28.22	15.56	27.83	22.94		21.85	8.27	2.93
(Ipsilateral Values Only)	2	13.60	16.73	18.07	20.48	8.87	18.10	16.65	18.36	18.00		16.54	3.41	1.21
	3	15.82	17.46	17.65	19.78	12.71	18.51	17.01	18.24	18.20		17.26	2.02	0.72
	4	17.56	20.29	18.04	19.85	13.67	19.00	16.14	19.23	17.27		17.89	2.06	0.73
	5	18.32	18.88	17.65	20.93	14.30	20.02	16.47	20.78	17.01		18.26	2.18	0.77
	6	29.31	27.38	29.54	38.98	22.52	36.21	29.05	36.48	32.37		31.32	5.19	1.84
	7	16.76	19.16	21.90	28.45	9.14	29.66	15.92	27.03	26.39		21.60	6.90	2.44
	8	5.74	16.45	13.97	18.25	1.53	20.43	3.68	18.00	15.16		12.58	7.03	2.48
SUB-TOTAL	I	131.80	159.51	163.06	198.83	88.68	190.16	130.47	185.94	167.33		157.31	35.08	12.40
	C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00
TOTAL		*131.80*	*159.51*	*163.06*	*198.83*	*88.68*	*190.16*	*130.47*	*185.94*	*167.33*		*157.31*	*35.08*	*12.40*
STRIATUM (mm ^{3})	1	1.60	1.66	4.20	4.30	0.37	3.68	2.31	2.98	2.76		2.65	1.31	0.46
(Ipsilateral Values Only)	2	4.99	6.67	7.71	6.49	2.94	7.63	6.61	6.53	6.43		6.22	1.46	0.52
	3	11.15	11.28	12.57	6.24	8.60	12.71	12.41	11.87	11.15		10.89	2.14	0.76
	4	13.13	11.50	13.13	4.65	10.89	13.08	13.13	13.00	12.27		11.64	2.75	0.97
	5	8.60	8.50	8.68	5.19	7.55	8.58	8.61	8.68	8.63		8.11	1.15	0.41
	6	4.86	5.26	5.41	5.28	5.41	5.25	5.27	5.41	5.32		5.27	0.17	0.06
	7	0.00	0.40	0.40	0.40	0.40	0.39	0.40	0.40	0.40		0.35	0.13	0.05
	8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00
SUB-TOTAL	I	44.34	45.28	52.10	32.56	36.16	51.32	48.73	48.87	46.97		45.15	6.68	2.36
	C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00
TOTAL		*44.34*	*45.28*	*52.10*	*32.56*	*36.16*	*51.32*	*48.73*	*48.87*	*46.97*		*45.15*	*6.68*	*2.36*
MID-LINE (mm ^{3})	1	0.00	1.86	0.28	0.97	0.02	1.06	0.00	0.15	0.01		0.48	0.66	0.23
(Non Str/Cort Regions-	2	0.81	2.59	0.59	0.44	0.01	1.32	0.14	0.22	0.02		0.68	0.83	0.29
Ipsilateral Values Only)	3	2.07	2.75	0.55	0.01	0.00	1.38	0.63	0.33	0.03		0.86	0.99	0.35
	4	5.76	4.65	3.12	0.01	0.04	5.05	2.62	1.86	5.00		3.12	2.17	0.77
	5	8.39	9.78	4.48	4.19	1.64	10.51	2.21	4.78	9.02		6.11	3.35	1.19
	6	9.97	26.83	3.05	19.95	6.79	26.17	0.57	10.07	13.64		13.00	9.51	3.36
	7	3.27	31.43	0.12	11.58	3.79	16.35	0.40	4.00	5.59		8.50	10.07	3.56
	8	1.09	36.05	0.00	0.00	0.21	2.24	0.00	0.00	0.00		4.40	11.89	4.20
SUB-TOTAL	I	31.36	115.92	12.18	37.15	12.50	64.08	6.58	21.40	33.31		37.16	34.27	12.11
	C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00
TOTAL		*31.36*	*115.92*	*12.18*	*37.15*	*12.50*	*64.08*	*6.58*	*21.40*	*33.31*		*37.16*	*34.27*	*12.11*
IPSILATERAL		207.51	320.71	227.34	268.54	137.34	305.57	185.78	256.22	247.60		239.62	76.02	26.88
CONTRALATERAL		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00
OVERALL		*207.51*	*320.71*	*227.34*	*268.54*	*137.34*	*305.57*	*185.78*	*256.22*	*247.60*		*239.62*	*57.71*	*20.40*
OEDEMA (%)		*23.00*	*42.00*	*26.00*	*30.00*	*20.00*	*45.00*	*22.00*	*32.00*	*33.00*		*30.33*	*8.73*	*3.09*
Experiment Ref		JMX1005	JMX1005	JMX1007	JMX1007	JMX1010	JMX1015	JMX1012	JMX1012	JMX1016				
Data File		PMF98	PMF98	PMF98.1	PMF98.1	PMF98.2	PMF98.3	PMF98.3	PMF98.3	PMF98.3				
Oedema File		OEDEMA1	OEDEMA1	OEDEMA1	OEDEMA1	OEDEMA1	OEDEMA1	OEDEMA1	OEDEMA1	OEDEMA1				

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